INVESTIGATIONS ON GLUCOSE CATALYSIS IN BACTERIAL METABOLISM

II. PYRUVATE AND GLUCOSE METABOLISM IN CELL-FREE EXTRACTS OF *LACTOBACILLUS ARABINOSUS 17–5*

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Previous studies on the metabolism of pyruvate in whole cells of *Lactobacillus arabinosus 17–5* have shown that added pyruvate is converted quantitatively to acetoin and CO_2 (Nossal, 1952), according to the reaction

$$2 \text{ pyruvate} \rightarrow \text{acetoin} + 2\ CO_2$$

The overall reaction is stimulated by concurrent glycolysis (Hunt and Nossal, 1954), but the decarboxylation of acetolactate, an assumed intermediate in the formation of acetoin, is not so stimulated.

The present paper is concerned with the study of these reactions in cell-free extracts, where, in contrast to whole cells, acetoin was not the sole product of pyruvate metabolism. Aerobically, a pyruvate oxidation system could also be demonstrated. This system generated acetate and acetyl phosphate in the presence of phosphate. The *Lactobacillus* system is the reverse of that described by Dolin and Gunsalus (1951) for *Streptococcus faecalis* in which whole, dried cells oxidised pyruvate whereas extracts formed acetoin. Our overall system resembles the combined acetoin and oxidase systems in *Aerobacter aerogenes* and *Staphylococcus aureus* (Strecker and Ochoa, 1954), but differs markedly in several respects. The phosphate-dependent oxidative activity in *Lactobacillus arabinosus* extracts is probably similar to the system in *Lactobacillus delbruckii* (Hager, Geller and Lipmann, 1954), and the phosphate independent oxidase to the system in *Proteus vulgaris* (Moyed and O'Kane, 1952). The overall oxidase activity resembles the dual system indicated by Razzel and Gunsalus (1954) for *Escherichia coli*.

Methods and Preparations.

Medium. Except where variations in culture conditions were studied, cells used in these experiments were grown on the same *Pronutrin-glucose* medium as in the previous paper, with the further addition of 80–100 ml./l. of a yeast extract prepared by boiling Effront baker's yeast with an equal volume of water and filtering. Sodium citrate was used as a buffer to replace sodium acetate, the former giving better cell yields and higher extract activities. Cells were harvested, washed, and stored as previously described. Maximum cell-extract

activities were obtained only when the "high yeast extract" (H.Y.E.) medium was employed. There was little or no activity in extracts from cells grown in Pronutrin with low yeast extract (L.Y.E.) content (10–20 ml./l.) or in a variety of other casein hydrolysates, peptones and tryptones, with high or low yeast extract content.

Estimations. Acetoin by the method of Westerfeld (1945); acetate according to the microdistillation method of Bartley (1953) with modifications similar to those described by Von Korff (1954); acylphosphates by the hydroxamic acid method of Lipmann and Tuttle (1945); organic acids by the silica-gel column chromatographic procedure described by Ladd and Nossal (1954); glucose and total carbohydrate after the anthrone method of Trevelyan and Harrison (1952); phosphorylated glycolytic intermediates spectrophotometrically, using the rabbit muscle enzyme fraction procedure of Slater (1953). Gas exchange was followed with standard Warburg equipment at 30° C.

Reagents and Enzymes. Lactic dehydrogenase was obtained as a purified rabbit muscle fraction according to Kornberg and Pricer (1953). A purified muscle fraction precipitating above 72 p.c. ammonium sulphate saturation was used as a source of enolase. Phosphoenolpyruvic kinase was a preparation kindly donated by Dr. M. F. Utter. Diphosphothiamine was synthesized by the method of Karrer and Visconti (1946). Hexose esters and all inorganic reagents used were of the purest grade available. α-Lipoic acid was a preparation from Lederle Laboratories, coenzyme A the 1 p.c. Sigma preparation, and DPN Sigma "65." Reduced DPN was prepared by hydrosulphite reduction according to Ohlmeyer (1938).

Preparation of the extract. Cell-free extracts were prepared by disintegration of the cells with glass beads at 0–2° C. in the high-speed shaker described by Nossal (1953). Shaking frequencies, and times of disintegration, varied as described in the text, but generally frequencies of 5,600 r.p.m. for 40 seconds or 3,000 r.p.m. for 90 seconds were used. Routinely, the capsules were charged with 15 ml. cell suspension containing approximately 20 mg. cells dry weight/ml. in 0·066 M potassium succinate buffer, and 10 gm. Ballotini No. 14 beads. Glass and cell debris were removed by centrifugation in the cold at 3,000 *g.* for 20 minutes. The cell-free supernatant, designated whole extract (W) in the text, was further centrifuged for 20 minutes at 0° C., at 20,000 *g.* in the Sorvall refrigerated centrifuge yielding a particulate residue (R), and a water-clear straw-yellow coloured supernatant (S.).

RESULTS.

Pyruvate decomposition by whole extracts.

Succinate was the only buffer suitable for studying the basic activity of whole and fractionated extracts with pyruvate as substrate. Citrate was oxidatively decarboxylated by the extracts, although unattacked by whole cells. Acetate and phosphate are implicated in the enzymatic reaction and are unsuitable for this reason, whilst other buffers were inhibitory or have pH ranges beyond that required for optimal pyruvate decomposition. In W, diphosphothiamine and manganese ions were required for maximum rates of pyruvate decomposition (Table 1). Magnesium and cobalt ions, but not nickel, replaced manganese. Aerobically, at pH 6·0, pyruvate was partly converted to acetoin and CO_2 and partly oxidised. The oxidative reaction was greatly increased by the addition of inorganic phosphate. Phosphate was effective in this reaction at varying concentrations and at pH 5·2–6·6. Anaerobically, pyruvate was converted wholly to acetoin and CO_2, the rate of CO_2 evolution being much less than the aerobic

TABLE 1.

General Properties of the Whole Extract.

	Basal System	+30 μmols PO_4^{---}	$-Mn^{++}$	$-Mn^{++}$ −cocarboxylase	$-Mn^{++}$ $+Ni^{++}$	$-Mn^{++}$ $+Co^{++}$	$-Mn^{++}$ $+Mg^{++}$	Basal system Anaerobic
μl CO_2	147	276	100	61	100	150	147	67
μl O_2	40	110	30	20	30	40	40	0

Cups contain in the basal system: 10 μmoles metal chloride, 30 μmoles sodium pyruvate, 300 μg. cocarboxylase, 0·066M potassium succinate buffer pH 6·0, 1·0 cc whole extract from 40 sec., 5,600 R.P.M. disintegration, added to iced cups. Protein 4·6 mg. Gas phase air, or nitrogen where indicated. Reaction time 60 min.

rate. Variations in the rates and ratios of CO_2 output and oxygen uptake occurred with variations in substrate concentration. Table 1 shows the activity of the extract using final pyruvate concentration of 1×10^{-2} molar.

Stoichiometry of the reaction.

Under all conditions, oxygen uptake with and without phosphate was balanced by the formation of equivalent amounts of acetate as measured by volatile acid distillation. A quantitative CO_2 balance, however, was not always obtained. The "acetoin CO_2", or that quantity of CO_2 remaining after correcting the total CO_2 evolution for oxidative decarboxylation, was balanced by either a 100 p.c. equivalent, or an approximately 66 p.c. equivalent of acetoin formation. The mechanism of acetoin formation appears to involve α-acetolactate in these extracts. According to Kobayashi and Kalnitsky (1954), *Proteus morganii* utilizes 3 molecules of pyruvate per molecule of acetolactate formed, the ratio being altered to 2:1 by added cocarboxylase. Therefore, in this system also, either two or three molecules of pyruvate disappear for one molecule of acetoin produced. The cocarboxylase added in all experiments, however, remained constant. The fate of the third pyruvate molecule remains unknown. No volatile bisulphite-binding compounds could be detected in the reaction mixtures.

Relationship between cell activity, glucose effect, and extract activity.

No significant difference was obtained in the pH of the media containing high or low yeast extract at the time of harvesting the cells. Final pH values were between 4·6–5·0, but no correlation between pH and enzyme activity was found, contrary to the observations of Moat and Lichstein (1953). A variety of growth factors, including thiamine, riboflavin, pantothenic acid, nicotinic acid, glutamine, purine and pyrimidine compounds, failed to duplicate the effect of the high yeast extract addition to the growth medium. In one or two experiments, increased biotin levels gave cells with higher activities. This response was irregular and in no case as great as with yeast extract. The increased rate of

pyruvate decomposition in these optimal cell extracts was manifested as an increase in the acetoin reaction only. The rates of pyruvate oxidation remained relatively unchanged in the high or low yeast extract grown cells. This applied also to the acetolactate decarboxylase reaction. Assuming that acetoin production passes through acetolactate in this system, the increased acetoin activity of optimal extracts is then due to an increase in the condensation reaction forming acetolactate. These relationships are illustrated graphically in Fig. 1.

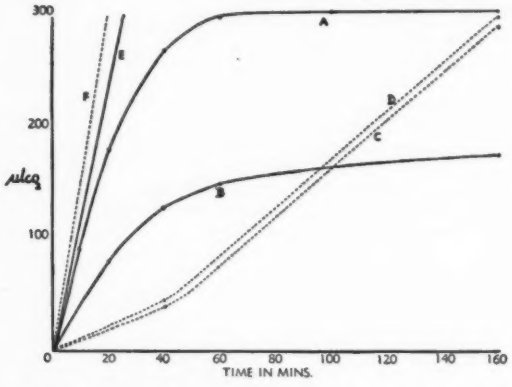

Fig. 1. Activities of extracts from cells grown in high and low yeast extract media.
Curve A: Acetoin CO_2 H.Y.E. media.
Curve B: Acetoin CO_2 L.Y.E. media.
Curve C: Oxidative CO_2 H.Y.E. media.
Curve D: Oxidative CO_2 L.Y.E. media.
Curve E: Acetolactate CO_2 H.Y.E. media.
Curve F: Acetolactate CO_2 L.Y.E. media.
Warburg cups contained the basal system as in Table 1 with the addition of 30 μmoles phosphate. Curves E and F 30 μmoles DL-acetolactate replaced pyruvate. Cells from high (H.Y.E.) and low (L.Y.E.) yeast extract media were adjusted to approx. 20 mgm. dry weight per ml., and disintegrated for 40 secs. at 5,600 r.p.m.

Generally the activity of extracts could be correlated with the basic activity, but not with the glucose-stimulated activity, of the whole cells. Some cell batches grown in low yeast extract media had very poor basic whole cell activities which could be abolished completely by short incubation of the cells in the Warburg cups, but the glucose-stimulated activity remained high and constant (Fig. 2). Such cell batches invariably gave extracts which failed to convert pyruvate to acetoin, although possessing acetolactate decarboxylase and suboptimal pyruvic oxidase activity. Results of this type indicate no lack of pyruvate apoenzyme in poor cells, but rather of some cofactor requirement. This cofactor is also lacking in the cell-free extracts, but is maintained by glycolysis in the whole cells.

Relation of activity and pH.

Whole extracts showed an unusual pH plateau for pyruvate decomposition. The optimal pH was 6·0, but the rates of CO_2 evolution (corrected for absorption) and O_2 uptake varied only slightly between pH 5·0 and 7·0. Acetoin production followed the same curve. Beyond this range both activities decreased sharply. At pH 4·5 and 7·5 both systems were completely inactive. The system

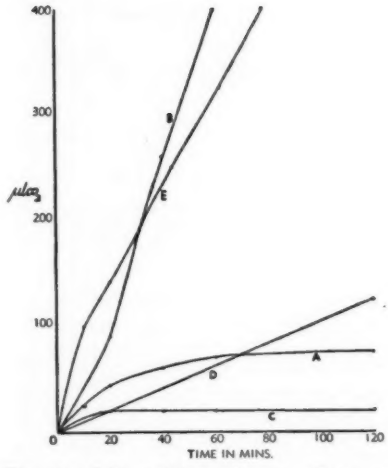

Fig. 2. Cell activity, glucose effect, and extract activity relationships.

Curve A: Whole cells, no glucose.
Curve B: Whole cells and glucose.
Curve C: Whole extract acetoin CO_2.
Curve D: Whole extract oxidative CO_2.
Curve E: Whole extract acetolactate CO_2.

For whole cell activities, cups contained 30 μmoles $MnCl_2$, 10 μmoles KCl, 30 μmoles sodium pyruvate, 0·066 M citrate-phosphate buffer pH 3·8, and 30 μmoles glucose where indicated. Gas phase air. 4·0 mgm. cells dry weight. Whole extract activities as in Fig. 1.

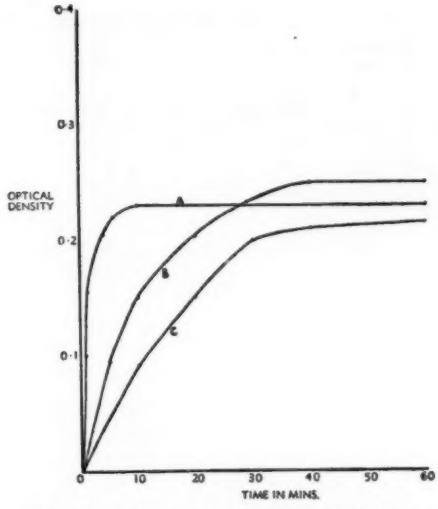

Fig. 3. Time curves of colour development for acetoin, diacetyl, and the product of pyruvate decarboxylation.

Curve A: 10 μg. diacetyl.
Curve B: pyruvate decarboxylation product.
Curve C: 10 μg. acetoin.

responsible for increased oxidative decarboxylation in the presence of phosphate was more active in the lower pH range 5·2 to 5·5.

Reaction time curves and the effect of substrate concentration.

During the aerobic metabolism of pyruvate in the presence and absence of phosphate, marked variation in the rates of the individual reactions occurred. At pH 5·2 in the presence of phosphate, and with 'final' pyruvate concentration of 1×10^{-2} M, oxidative decarboxylation proceeded steadily to completion, whereas acetoin formation ceased after 30 minutes. In the absence of phosphate acetoin production continued for longer periods but ceased before completion of oxidative decarboxylation. Substrate concentration data are presented in Table 2. Little or no acetoin was found at low pyruvate concentration (0·003 M) in the presence of phosphate.

TABLE 2.

The Effect of Added Coenzymes and Acceptors in Whole Extracts.

Expt. (1).

	No Additions	+30 μmols Acetate	+30 μmols Acetaldehyde	Acetate Alone	Acetaldehyde Alone	+30 μmols Ferricyanide
μl CO_2	422	450	430	0	0	450
μl O_2	100	120	113	0	0	0
μmols Acetoin	4·0	4·5	4·5	0	0	0

Cups contained 10 μmoles $MnCl_2$, 30 μmoles sodium pyruvate, 300 μg. cocarboxylase, 0·066M potassium succinate buffer pH 6·0. Other additions as indicated. 1·0 cc whole extract from 40 sec., 5,600 R.P.M. disintegration, added to iced cups at zero time. Protein 4·0 mgm. Gas phase air. Reaction time 60 min.

Expt. (2).

	No additions	−Phosphate	+CoA	+CoA+DPN	+CoA+DPN +α-Lipoic
μl CO_2	500	400	510	495	500
μl O_2	140	81	135	140	140
μmols Acetoin	3·8	3·8	3·8	3·8	3·8

Cup additions as above, but with 30 μmoles potassium phosphate pH 6·0. Where indicated, 300 μg. 1 p.c. CoA, 300 μg. 65 p.c. DPN, 100 μg. α-lipoic acid.

Effect of added coenzymes and acceptors on pyruvate decomposition in whole and dialysed extracts.

In view of the extensive evidence for the requirements of DPN, CoA, and α-lipoic acid in most pyruvate metabolizing systems, the effect of various cofactors was tested. None of the above compounds caused any stimulation of pyruvate utilization in whole extracts. In extensively dialyzed extracts, oxidation with and without phosphate was independent of added coenzymes. Treatment of extracts with Dowex-1 (chloride form, 200–400 mesh) also failed to reveal any cofactor requirements. Acetaldehyde, acetate, and acetyl phosphate in combination with the above cofactors did not cause any increase in acetoin formation with pyruvate as substrate.

Ferricyanide was the only compound to affect these systems. Aerobically, addition of ferricyanide caused stimulation of CO_2 evolution, and complete suppression of oxygen uptake and acetoin production. Anaerobically, ferricyanide again caused stimulation of CO_2 evolution and suppression of acetoin formation. No other electron acceptors acted similarly. Methylene blue and a variety of redox dyes ranging in E_0 values at pH 7·0 from −0·416 to +0·224 failed to substitute for oxygen or to stimulate decarboxylation (Table 3). Similar results were obtained with partially purified preparations as described in a later section.

TABLE 3.

Substrate Concentration in Whole Extracts.

Pyruvate Concentration	No phosphate			+Phosphate		
	μl CO_2	μl O_2	μm acetoin	μl CO_2	μl O_2	μm acetoin
m/300	110	22	0·66	228	109	0
m/100	380	18	7·5	445	160	3·0
m/50	610	0	14·0	1060	204	14·0

Cup additions as in the basal system with pyruvate concentration as indicated. Gas phase air. Reaction time 120 min.

Nature of "acetoin" formed and the metabolism of diacetyl in whole extracts.

The alkaline α-naphthol creatine reagent for the estimation of acetoin and diacetyl differentiates between these two compounds by the time required for complete colour development. Full colour is attained with diacetyl in less than 10 minutes, while acetoin requires 60 minutes. Although the product formed by pyruvate decarboxylation in whole cells of *Lactobacillus arabinosus,* reacts chemically as acetoin (Nossal, 1952), some doubt exists on the nature of the compound formed by the non-oxidative decarboxylation of pyruvate in whole extracts of the organism. The time curve of colour development follows a line in between that of acetoin and diacetyl (Fig. 3). It is possible that this time curve is a composite one of both acetoin and diacetyl, but no evidence for the interconversion of these compounds could be demonstrated in the extracts.

In *A. aerogenes,* Strecker and Harary (1954) found enzymes catalyzing the reversible oxidation of butylene glycol to acetoin and the reduction of diacetyl to acetoin. Both of these enzymes were DPN-linked. The diacetyl mutase reaction of Green (1947), in which oxidation of one molecule of diacetyl gives rise to two molecules of acetate with reduction of a second molecule of diacetyl to acetoin, was considered to involve the diacetyl reductase according to these workers. The *Lactobacillus* system, however, does not appear to possess such enzymes. In the Warburg cups, diacetyl was utilized very slowly, giving out CO_2 and taking up oxygen at rates little higher than those of the blank. Under the same conditions, pyruvate was used rapidly. Spectrophotometrically, no reoxidation of DPNH or reduction of DPN occurred with either diacetyl or acetoin as substrates. The possibility remains that both diacetyl and acetoin are produced simultaneously from pyruvate by different pathways, accounting for the anomalous colour-time curve. Investigations are continuing along these lines.

Some doubt also exists concerning the nature of the products formed by the action of neutral phenylhydrazine on acetolactate, diacetyl and acetoin. Juni (1952) reported that acetolactate formed the osazone of diacetyl (M. pt. 248° C.),

diacetyl gave the monohydrazone (137° C.), while acetoin did not react. Kobayashi and Kalnitsky (1954) found all three compounds gave the same derivative. The author's experience confirms the results of Juni.

Distribution of enzyme activity in the extracts.

All previous studies on pyruvic oxidase and acetoin-forming systems have been carried out in soluble systems. Strecker and Ochoa (1954) recently described the separation and partial purification of these combined systems from *Aerobacter aerogenes* and *Staphylococcus aureus*. No evidence has been found for an acetoin-forming system in organized particulate fractions of the cell. By high-speed centrifugation of the whole extracts prepared under the present conditions, a particulate fraction could be obtained which converted pyruvate to acetoin and CO_2. The oxidative activity of W remained in supernatant. The distribution of these activities is shown in Table 4. High-speed centrifugation

TABLE 4.

Distribution of Activity.

	No phosphate		+Phosphate	
	μl CO_2	μl O_2	μl CO_2	μl O_2
Whole Extract	560	69	640	140
Supernatant	515	68	610	160
Particulate Fraction	460	10	460	10

Cup additions as in the basal system, Table 1, with 30 μmoles potassium phosphate where indicated. 1·0 ml. whole extract, 90 sec, 3,000 R.P.M. disintegration; 1·0 ml. supernatant; 1·0 ml. particulate fraction obtained by resuspending the residue from 20 ml. of W in 4 ml. disintegration medium, added to iced cups as indicated. Gas phase air. Reaction time 120 min.

thus provides a means of obtaining an acetoin-forming system free of oxidative activity. Such results also indicate the presence of at least two separate pyruvate-decomposing enzymes in W. This is in contradistinction to previous ideas of a single pyruvate apoenzyme generating a common intermediate for

TABLE 5.

Effect of Substrate Concentration in the Particulate Fraction.

Pyruvate Concentration	No Phosphate		+Phosphate	
	μl CO_2	μl O_2	μl CO_2	μl O_2
m/300	76	0	76	0
m/100	220	0	220	0
m/50	320	0	320	0

Cup additions as in Table 4, with sodium pyruvate concentration varied as indicated. Reaction time 120 min.

acetate or acetoin formation. Substrate concentration studies confirm the differences between W and R. W oxidized pyruvate with little or no acetoin formation below substrate concentrations of 0·003 M. R forms only acetoin regardless of substrate concentration (Table 5).

Fractionation of whole extracts.

W, S, and R all decomposed acetolactate rapidly at pH 6·0. However, it has not been possible to isolate and identify acetolactate as an intermediate from pyruvate in these systems. All attempts to remove acetolactate decarboxylase completely from fractionated extracts were unsuccessful. With S, prepared by 90-second slow-speed disintegration and adjusted to pH 5·8 with M succinic acid, treatment with calcium phosphate gel in the cold removed all the acetoin-forming capacity, leaving 90 p.c. of the oxidative system and the acetolactate decarboxylase activity in the supernatant. As previously described by Juni (1952), the acetoin-forming system could be eluted from the gel at a higher pH. In this system 0·05 M trishydroxyaminomethane-succinate buffer pH 8·3 was used for elution. The supernatant obtained after centrifuging the eluted gel was adjusted to pH 6·0 with M succinic acid. This fraction with addition of cocarboxylase and manganous ions decarboxylated pyruvate readily, but also decomposed acetolactate at 10 p.c. of the rate of the original supernatant. Repeat treatment with calcium phosphate gel gave final eluates very low in protein content and of variable activity. Active preparations of this fraction decarboxylated pyruvate but not added acetolactate. However, pyruvate decomposition gave rise to acetoin. The yield of acetoin was not increased on subsequent incubation of the reaction mixture with aniline citrate at pH 4·5.

In *A. aerogenes* Juni (1952) was able to separate the acetolactate-forming and decarboxylating fractions by ethanol fractionation of the calcium phosphate gel eluate. With *Lb. arabinosus* extracts, entirely different results were obtained, using methods similar to those described by Juni. 90 ml. S were adjusted to pH 5·8 with M succinic acid and 20 ml. calcium phosphate gel (containing 25 mg. gel/ml.) were added. The mixture was centrifuged at low speed of 0° C. after standing 10 minutes. The gel was washed with 20 ml. of the cell disintegration medium, and finally eluted twice with a total volume of 50 ml. of tris-succinate buffer pH 8·3. The eluate was adjusted to pH 6·0 with M succinic acid, 45 ml. of ethanol (—15° C.) were added slowly with stirring, keeping the mixture at —5° C. with the aid of an ice-salt bath. Precipitation was allowed to proceed at —15° C. overnight. The white precipitate was centrifuged at 8,000 *g.* at —15° C. for 10 minutes and the supernatant was discarded. The precipitate was then triturated at 0° C. with 12 ml. 0·06 M succinate buffer pH 6·0.

Practically all the material redissolved (cf. *A. aerogenes* fraction of Juni). The opalescent solution was centrifuged at 0° C. and 8,000 *g.* for 10 minutes, yielding a small insoluble fraction and a water-clear supernatant. In contrast

to *A. aerogenes*, acetolactate was rapidly decomposed by this *Lactobacillus* fraction. Pyruvate was also decarboxylated in the presence of cocarboxylase and manganous ions and only acetoin was formed. The activity with pyruvate proved to be unstable. In freshly prepared fractions, CO_2 was evolved rapidly for 10–20 minutes, but ceased after 30 minutes. This activity could be restored completely by the addition of small quantities of cysteine. Pyruvate was not attacked at all by fractions aged at 0° C. for more than four hours from the time of redissolving. Cysteine restored the activity of these preparations.

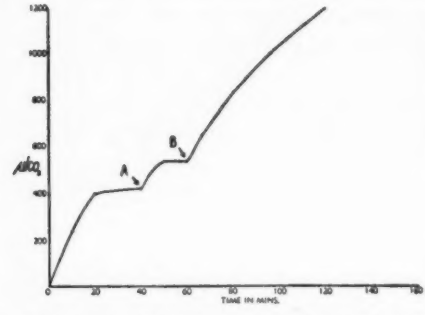

Fig. 4. Activity of the ethanol precipitated gel eluate. Cup additions as in Fig. 1 except 1·0 ml. redissolved ethanol precipitated fraction added to iced cups and 50 μmoles sodium pyruvate. At point A, a further 0·5 ml. enzyme was added, and at point B, 10 μmoles cysteine. Gas phase air.

In later experiments, glutathione was found to be many times more effective than cysteine in stimulating acetoin formation in fresh and for reactivating aged preparations. Added coenzyme A, lipoic acid, and DPN-TPN were not required for optimal rates of decarboxylation in the presence of cysteine or glutathione. Usually, three molecules of pyruvate were utilized for the production of one molecule of acetoin. Diacetyl was not metabolized. Unlike whole extracts, pyruvate decomposition was restricted to a narrow pH region, optimal at pH 6·0 and inactive beyond pH 5·5 and 6·5. The preparation was devoid of oxidative activity in the presence or absence of phosphate. Decomposition of pyruvate and acetolactate is shown in Table 6 and Fig. 4.

TABLE 6.

Pyruvate Metabolism by the Gel Eluate Ethanol Fraction.

	pH 6·0				pH 5·2	
	No Additions	Cysteine	+Cysteine No Pyruvate	Aceto-Lactate No Pyruvate	No Additions	+ Cysteine
μl CO_2 20 min	167	440	0	336	0	60
μl CO_2 60 min	167	930	0	—	12	80
μl O_2 60 min	0	0	0	0	0	0

Cups contained 10 μmoles $MnCl_2$ 50 μmoles sodium pyruvate, 300 μg. cocarboxylase, 0·06M potassium succinate buffer pH 6·0 or 5·2, 10 μmoles cysteine and 30 μmoles DL-α-acetolactate where indicated, 1·0 ml. cold freshly dissolved ethanol fraction added to iced cups at zero time. Gas phase air.

Nature of pyruvate oxidation in fractionated extracts.

The supernatant fraction remaining after treatment of S with calcium phosphate gel at pH 5·8, contained the pyruvate oxidation system free from the acetoin-forming reaction. This provided an ideal system to study the nature of pyruvate oxidation without the complication of condensation reactions. As in W, addition of phosphate caused marked stimulation of oxygen uptake. Treatment of these reaction mixtures with hydroxylamine and ferric ions according to Lipmann and Tuttle (1945) gave the characteristic purple colouration indicative of acyl phosphate. No acyl phosphate was generated by pyruvate oxidation in the absence of added phosphate. Oxidation was accompanied by an equivalent formation of acetate. Quantitative acyl phosphate estimations using succinic anhydride standards, showed that the additional oxygen uptake when phosphate was added resulted in an equivalent formation of acetyl phosphate. As in untreated extracts, addition of DPN, CoA, and α-lipoic acid, failed to stimulate pyruvate oxidation. Dialysis, followed by Dowex 1 (CL form, 200–400 mesh) treatment, although removing the yellow colour of the extract, again revealed no added cofactor requirement. In fact, addition of 300 μg. Sigma 1 p.c. coenzyme A preparation depressed acetyl phosphate formation. Unlike the acetoin-forming ethanol fraction, cysteine and glutathione did not increase the rates of oxidation, even in dialyzed, Dowex-treated fractions, and could reactivate aged preparations. Estimations of bound DPN, CoA and lipoic acid are in progress. Unless these estimations prove to be positive, pyruvate oxidation in our extracts appears to involve a pathway not requiring these cofactors. The mechanism of electron transport is unknown. Pyruvate oxidation in this fraction is shown in Table 7.

TABLE 7.

Pyruvate Oxidation in Gel Treated Extracts.

	No additions	+ phosphate	+phosphate +cysteine	+phosphate +cysteine +cofactors
μl CO_2	300	470	470	463
μl O_2	150	230	230	243
μm. total volatile acid (acetic+acyl phosphate)	15	23	23	23·6
μm. acyl phosphate (hydroxamate)	0	8·0	8·0	5·2

Cup additions as for basal system Table 1, with 30 μmoles phosphate, 10 μmoles cysteine, and cofactor solution containing 30 μg. α-lipoic, 300 μg. 1 p.c. CoA, 300 μg. 65 p.c. DPN, added where indicated.

Glycolysis in crude extracts.

In whole cells of the organism, pyruvate decomposition is stimulated by concurrent glycolysis. Addition of glucose to crude extracts metabolizing pyruvate caused no increase in the rate of CO_2 evolution. If the glucose stimu-

lation in whole cells is a cell-wall permeability phenomenon, no glucose effect could be expected in cell-free extracts. Since catalysis in whole cells is produced only during concurrent glycolysis of the added sugar, an incomplete glycolytic system in extracts could also result in failure to stimulate pyruvate decomposition. The metabolism of carbohydrate in extracts was therefore further investigated. Crude extracts contained considerable quantities of soluble carbohydrate as measured by the anthrone technique. Chromatographic analysis of the organic acid content by the method of Ladd and Nossal (1954) showed the presence of only one acid. This appeared between fractions 88–95, and was identified as lactic acid. It was not possible to estimate lactic acid in extracts using the procedure of Barker and Summerson (1941), due to the presence of very large quantities of non-dialysable compounds which produced intense colour reactions with sulphuric acid and *p,p'*-dihydroxydiphenyl.

Glucose metabolism in whole extracts was studied in two ways. The method of Slater (1952) for estimation of hexose mono- and di-phosphates was used to study the formation of these esters from glucose, and to measure their rate of utilization when added to substrates. The enolase, phosphoenolpyruvic kinase, and lactic dehydrogenase activities of extracts were studied spectrophotometrically by measuring the rate of disappearance of DPNH at 340 mμ in the Beckman spectrophotometer, using appropriate substrates. Results for glucose and hexose phosphate utilization typical of many experiments are presented in Table 8.

In no experiments could any utilization of hexose beyond the triose phosphate stage be demonstrated. Results of this type were obtained for extracts from high and low speed disintegrated cells. The addition of cysteine, Mn^{++}, Co^{++}, Zn^{++} (all 1×10^{-3} M) in various combinations with and without versene

TABLE 8.

Utilization of Glucose and Hexose Esters by Whole Extracts.

Incubation mixtures contained 16-20 μmoles substrate, 3·0 μmoles $MgCl_2$, 30 μmoles K.Na phosphate buffer pH 6·0, 24 μmoles A.T.P., 24 μmoles A.D.P., 180 μg. DPN, distilled water to 4·0 ml. Reaction commenced by addition of 1·0 ml. whole extract. Zero time controls obtained by removing 1·0 ml. reaction mixture to 3·0 ml. 10 p.c. trichloracetic acid, as soon as enzyme added. Test system incubated 30 minutes at 30° C, reaction stopped as before. Hexose esters were estimated in suitable dilutions of the neutralized trichloracetic acid mixture after centrifugation.

Substrate added	Initial concn. μmoles	Glucose	Recovered after incubation (μmoles)	
			Hexose monophosphate	Total Hexose diphosphate +triose phosphate
Glucose	30	25	0·8	4·2
Glucose-1-PO_4	25	—	25	0
Glucose-6-PO_4	24	—	4	20
Fructose-6-PO_4	24	—	4	20
Fructose-1-6-PO_4	25	—	0	25

(1×10^{-3} M) to the disintegration media or incubation mixtures causes no variation in these results. This indicates all the enzymes of the glycolytic sequence down to the first oxidation step are active under these conditions in these preparations. No evidence for an active triose phosphate dehydrogenase could be obtained under the conditions used. Glucose-1-phosphate was not attacked. The extracts also possess a powerful adenosine triphosphatase, confirming the work of Clark and MacLeod (1954). This explains the low utilization of added glucose with the concentrations of ATP used. A very active DPN-dependent lactic dehydrogenase was present, as indicated by the rapid disappearance of DPNH in the presence of pyruvate. Phosphoenolpyruvate in the presence of ADP and Mg^{++} ions failed to cause oxidation of DPNH, indicating the absence of an active phosphoenolpyruvic kinase. Addition of this enzyme, prepared from animal sources, to the cuvettes caused a rapid decrease in optical density. With 3-phosphoglyceric-acid as substrate, oxidation of DPNH occurred when external phosphoenolpyruvic kinase was added. This indicates the presence of an active enolase and glyceromutase. No evidence is available for the existence of diphosphoglyceric kinase. These results are illustrated in Figs. 5a, b, c. Addition of glucose, the hexose esters, triosephosphate, phosphoglyceric acid and phosphoenolpyruvate to W caused no stimulation in the rate

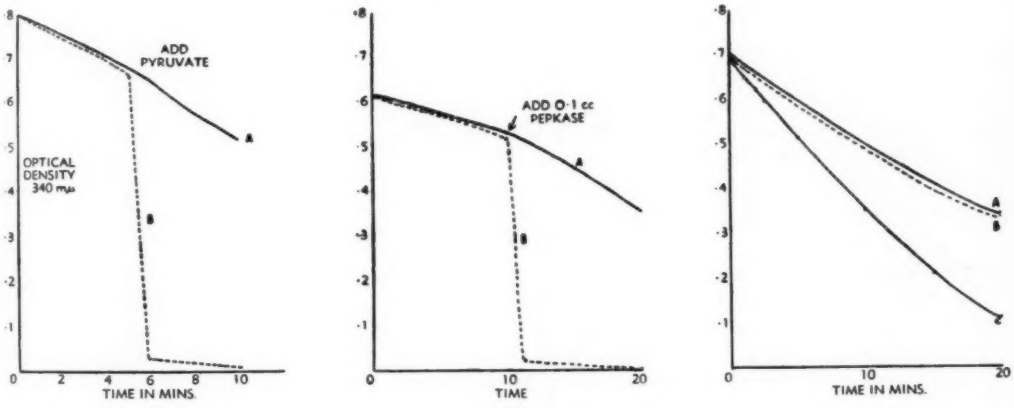

Fig. 5. (a) Lactic dehydrogenase: Quartz cuvettes contained 3 μmoles $MgSO_4$ 0·066 M potassium phosphate buffer pH 6·0, distilled water to 2·7 ml., and 0·4 μmoles DPNH. 0·2 ml. whole extract were added at zero time, and 0·3 μmoles sodium pyruvate as indicated.

(b) Phosphoenolpyruvate kinase: cuvettes additions as for (a) with 1·5 μmoles A.D.P. and 0·5 μmoles sodium phosphoenolpyruvate replacing sodium pyruvate. 0·2 ml. whole extract were added at zero time and 0·1 ml. of a $^1/_{40}$ dilution of crystalline phosphoenolpyruvate kinase as indicated.

(c) Enolase: cuvette additions as for (b) with 0·5 μmoles sodium 3-phosphoglycerate instead of phosphoenolpyruvate. 0·2 ml. whole extract and 0·1 ml. phosphoenolpyruvate kinase were added at zero time.

of pyruvate utilization. Addition of substrate-level quantities of DPNH resulted in decreased pyruvate decomposition, presumably due to conversion of pyruvate to lactate.

Clark and MacLeod (1954) recently described a complete glycolytic system in *Lb. arabinosus* cell free extracts prepared by grinding the cells with powdered glass. Glycolysis was followed by CO_2 evolution from a bicarbonate sodium phosphate medium in an atmosphere of CO_2. These results have been confirmed in this laboratory. However, under these conditions, pyruvate was not metabolized to acetoin. As yet, it has not been possible to obtain conditions under which glycolysis is complete, and pyruvate is converted to acetoin.

DISCUSSION.

Although the pathways of pyruvate metabolism both in microorganisms and in higher animals have been well established, the data available on the nature of pyruvic oxidase and acetoin formation remain incomplete. Dolin and Gunsalus (1951) found that cell suspensions and vacuum-dried cells of *S. faecalis* oxidized pyruvate to acetate and CO_2, but cell-free extracts from these cells catalysed the breakdown of pyruvate predominantly to acetoin and CO_2, although substrate concentration and glutathione shifted the balance in favour of oxidation. In *Lb. arabinosus*, not only do the whole cells convert pyruvate solely to acetoin, but an oxidative pathway does not become apparent until the cells are disintegrated. Several new features characterize the study of this system, particularly the pathway and nature of oxidation and condensation, and the yeast extract growth phenomena. Available data do not favour the idea of a competitive common intermediate for acetoin and acetate formation, viz., (a) anaerobically, there is a marked decrease in the rate of pyruvate breakdown, (b) increased substrate concentration does not reveal a true competitive effect, (c) separation of the acetoin and oxidation systems. Evidence here indicates the existence of at least two and possibly three separate pathways of pyruvate breakdown. Unlike the pyruvic oxidase of Schweet (1951) and other workers, no acetoin is formed in the oxidase fraction of the *Lactobacillus* system. Furthermore, no oxidative activity accompanies the aerobic acetoin formation in the particulate fraction separated from whole extracts. This is evidence for separate pyruvate apoenzymes for oxidative decarboxylation and acetoin formation. The fact that increase in oxidative activity by phosphate can be quantitatively accounted for by acetyl phosphate formation, whereas in the absence of phosphate, oxidative activity yields the free acid, favours the existence of a third pyruvate apoenzyme which is inactive in the absence of phosphate. The existence of a common 2-carbon fragment in pyruvate oxidation which reacts with phosphate to form acetyl phosphate, or water to form acetate in the absence of phosphate is not supported by the quantitative formation of acetyl phosphate.

The mechanism of acetoin formation in the extracts is not yet clear, even with the aid of the purified condensing system free from oxidative activity. Evidence is in favour of the acetolactate pathway, on present results and on current theories, although some conflicting data exist, *viz.*, (a) colour development time curves which throw doubt on the nature of the "acetoin" formed, (b) acetoin formation in fractionated extracts apparently free of acetolactate decarboxylase, (c) lack of activity by furacin. Asnis and Glick (1954) found that furacin (5-nitro-2-furaldehyde-semicarbazone) inhibited acetoin formation in *E. coli* and *S. faecalis*. In an atmosphere of nitrogen, 5×10^{-4} M furacin caused 80 p.c. inhibition of the rate of acetoin formation. This inhibition was completely absent in air. Furacin did not inhibit the enzymatic decarboxylaton of acetolactate, indicating inhibition occurs in the postulated conversion of pyruvate to acetolactate and CO_2. In *Lb. arabinosus*, both with whole cells and without glucose, and in cell-free extracts, no similar inhibitions by furacin could be obtained. Preparation of a fraction which can metabolize pyruvate and not attack acetolactate is needed to clarify these anomalies.

There is no lack of data for SH requirements in oxidative decarboxylation where CoA and lipoic acid are essentially concerned. However, both added CoA and lipoic acid, as well as SH compounds, do not appear necessary for pyruvate oxidation in this system.

The greatly enhanced acetoin activity in extracts of cells grown in Pronutrin media with increased yeast extract content is of importance in the light of possible new cofactor requirements in acetoin condensation reactions. Only acetoin formation, and not pyruvate oxidation or acetolactate decarboxylation, responds to high yeast extract media. These data may be useful in correlating with the glucose effect. The present work also indicates the doubtful possibility of obtaining the catalytic glucose effect in cell-free extracts. Although a complete glycolytic system does not exist under conditions where pyruvate is converted to acetoin, intermediates including the substrates and products of the missing enzymes, fail to cause stimulation of pyruvate metabolism. It is certain at least that no product of glycolysis is responsible for the catalytic effect.

SUMMARY.

Cell-free extracts of *Lactobacillus arabinosus 17-5*, prepared by high-speed disintegration with glass beads, possess enzymes for pyruvate oxidation as well as acetoin formation. These systems have been separated by centrifugation and by calcium phosphate gel, ethanol fractionation procedures.

Extracts optimally active in the formation of acetoin could be obtained only from cells grown in media containing much yeast extract.

Evidence is presented for the existence of two oxidative enzymes, one phosphate-dependent and forming acetyl phosphate, one phosphate-independent

and forming acetate. No requirement for added DPN, CoA or α-lipoic acid could be demonstrated.

The possibility of the acetoin-forming system existing in particulate fractions of the cell is presented.

No stimulation of pyruvate decomposition by added glucose could be achieved in cell-free extracts.

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REFERENCES.

Asnis, R. and Glick, M. (1954): Bact. Proc., p. 99.
Barker, S. B. and Summerson, W. H. (1941): J. biol. Chem., 138, p. 535.
Bartley, W. (1953): Biochem. J., 53, p. 305.
Clark, J. A. and MacLeod, R. A. (1954): J. biol. Chem., 211, p. 531, p. 541.
Dolin, M. I. and Gunsalus, I. C. (1951): J. Bact., 62, p. 199.
Green, D. E., Stumpf, P. K. and Zarudnaya, K. (1947): J. biol. Chem., 167, p. 811.
Hager, L. P., Geller, D. and Lipman, F. (1954): Fed. Proc., 13, p. 734.
Hunt, A. L. and Nossal, P. M. (1954): Austral. J. exp. Biol., 32, p. 533.
Juni, E. (1952): J. biol. Chem., 195, p. 715.
Karrer, P and Visconti, M. (1946): Helv. Chim. Acta, 29, p. 711.
Kobayashi, Y. and Kalnitsky, G. (1954): J. biol. Chem., 193, p. 481.
Kornberg, A. and Pricer, W. E. (1951): *Ibid.*, 193, p. 481.
Ladd, J. N. and Nossal, P. M. (1954): Austral. J. exp. Biol., 32, p. 523.
Lipmann, F. and Tuttle, C. (1945): J. biol. Chem., 159, p. 21.
Moat, A. G. and Lichstein, H. C. (1953): J. Bact., 66, p. 324.
Moyed, H. S. and O'Kane, D. J. (1952): J. biol. Chem., 195, p. 375.
Nossal, P .M. (1952): Biochem. J., 50, p. 591.
Nossal, P. M. (1953): Austral. J. exp. Biol., 31, p. 583.
Ohlmeyer, P. (1938): Biochem. Z., 297, p. 66.
Razzell, W. E. and Gunsalus, I. C. (1954): Bact. Proc., p. 97.
Schweet, R., Fuld, M., Cheslock, K. and Paul, M. (1951): "A Symposium on Phosphorus Metabolism," Vol. I. Johns Hopkins Press.
Slater, E. C. (1953): Biochem. J., 53, p. 157.
Strecker, H. J. and Harary, I. (1954): J. biol. Chem., 211, p. 263.
Strecker, H. L. and Ochoa, S. (1954): *Ibid.*, 209, p. 313.
Trevelyan, W. E. and Harrison, J. S. (1952): Biochem. J., 50, p. 298.
Von Korff, R. W. (1954): J. biol. Chem., 210, p. 539.
Westerfeld, W. (1945): *Ibid.*, 161, p. 445.

MULTIPLICATION OF *H. PERTUSSIS* IN THE MOUSE LUNG FOLLOWING INTRANASAL INFECTION

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The method of intranasal challenge (Burnet and Timmins, 1937) was adopted in this laboratory a number of years ago as a means of estimating active or passive immunity in mice against experimental pertussis (North, Keogh, Anderson and Williams, 1939; North and Anderson, 1941; North, Anderson and Graydon, 1941; North and Anderson, 1942; Anderson and North, 1943; North, 1946; Keogh and North, 1948; Fisher, 1950; and Warburton and Fisher, 1951). Either a large challenging dose was used which killed 80–100 p.c. of the controls, protection being manifested by a larger proportion of survivors among the immunized, or a smaller (sublethal) dose and the reduction in the degree of lung infection estimated.

During the last few years, increasing difficulty was experienced in obtaining satisfactory results with the lethal type of intranasal challenge usually employed. This may have been due to lessened susceptibility of the mice in this laboratory (inbred from a small colony for over 25 years) to freshly isolated strains of *H. pertussis* (Fisher, 1953). Attention was therefore turned to the sublethal type of challenge and the work now described was undertaken in order to obtain more detailed information than that available (North, Keogh, Anderson and Williams, 1939; North and Anderson, 1941; North, Anderson and Graydon, 1941; North and Anderson, 1942; Anderson and North, 1943; North, 1946; Proom, 1947; Cooper, 1952), so as to make it possible to design vaccine potency assays and to compare the results of such assays with those using other methods of challenge.

Materials and Methods.

Strains. The virulent strains FP12 and P81 were used for intranasal challenge unless otherwise stated, and strain 18-323 for the intracerebral challenge. The strains were maintained in the lyophilized state. Fresh lyophilized cultures were opened once every 3–4 weeks, or, if available, organisms recovered from lungs or brains of infected mice were used.

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[1]Part of the preliminary work and the experiments involving the vaccine K205 were carried out at the Lister Institute of Preventive Medicine, Elstree, Herts, England, during the tenure of a Nuffield Dominion Travelling Fellowship in Medicine.

Cultures. Cultures for challenge were grown overnight at 36° C. on Bordet Gengou slopes containing 33 p.c. defibrinated sheep blood. Lung cultures for the determination of viable counts were made on Bordet Gengou plates containing 0·5 unit penicillin per ml., and in those used for the later part of the work, 1 part in 550,000 (w/v) of 4:4′-diaminodiphenylamine dihydrochloride (M. & B. 938) as well (Lacey, 1951; Nicholson and Turner, 1954; Lacey, 1954). The plates were incubated for 5 or 6 days before the counting of colonies.

Suspensions and dilutions. Suspensions of bacteria for challenge, suspensions of lungs for viable counts, and all dilutions of these were made in Cohen and Wheeler's (1946) medium which, in preliminary experiments, was found somewhat superior to casamino acid solution. Bacterial suspensions were made up to the opacity standard issued for pertussis suspensions by the National Institutes of Health, Bethesda, Md., U.S.A. Tenfold dilutions were made in 4·5 ml. fluid contained in 1 oz. screw-cap bottles, using a fresh 1 ml. pipette for each dilution. Viable counts on suspensions used for intranasal challenge were performed as described below.

Mice. White mice of the strain of these Laboratories were used. Those used for intranasal dosage (without preliminary immunization) weighed 18–22 gm.; those immunized weighed 15–18 gm. at the beginning of the experiment.

Immunization. The immunizing agents were diluted in saline. 0·2 ml. amounts of the suspensions were injected by the intraperitoneal route.

Vaccines. The following vaccines were used:—

E75—a vaccine grown in Cohen and Wheeler's (1946) medium. Portion of this vaccine was heated in a boiling water bath for two hours.

AIII—a vaccine prepared by the Michigan Department of Health.

K205—a vaccine prepared by the Lister Institute, Elstree, Herts, England. Portion of this vaccine was heated in a boiling water bath for 2 hours.

Challenge. Intranasal challenge was performed according to the method of Burnet and Timmins (1937); intracerebral challenge, according to the method of Kendrick, Eldering, Dixon and Misner (1947). Mice receiving lethal doses by either route were observed for 14 days after challenge.

Evaluation of viable counts in the lungs of mice. Lung cultures were performed 7 days after the administration of the challenge dose, unless otherwise stated. The mice were killed with coal gas, the thoracic viscera removed aseptically and transferred to 1 oz. screw-cap bottles containing 5 ml. Cohen and Wheeler's (1946) medium and 20–22 stainless steel balls $^3/_{16}$″ in diameter. The lungs of one mouse were homogenized in one bottle. The bottles were agitated for 10 minutes in the horizontal position in a reciprocating shaker moving parallel with the long axis of the bottles, running approximately at 250 cycles per minute and having a stroke of 3 inches.

The number of viable organisms in the lung homogenates was estimated by a modification of the technique of Miles and Misra (1938). Small loops were prepared by bending fine (33 gauge) platinum iridium wire once around the shaft of a 20 gauge hollow needle and twisting the terminal part of the wire several times around the stem. The loop was dipped in the bacterial suspension and discharged on the culture plate by gently rubbing over an area of approximately $\frac{1}{2}$″ in diameter till the meniscus in the loop was broken. The loops were calibrated by replicate parallel platings of dilutions of the same bacterial suspension, with a standard dropping pipette and with the loop to be tested. The loops were found to deliver volumes between 0·5 and 1 cubic millimetre; the method was slightly less accurate than the standard method using drops, but was considered satisfactory.

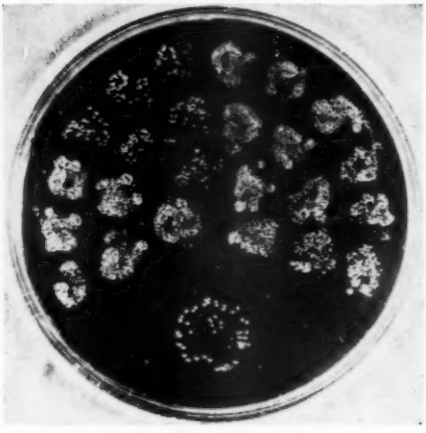

Fig. 1. Photograph illustrating method of viable counts by replicate plating with small loop.

The 4 sectors show platings of 4 lung homogenates, each in sixfold replicate. The colonies arranged over a circular area (below) are the plate control (one drop of a suitably diluted suspension of *H. pertussis*). Incubation for 5 days at 36° C.

Each suspension or dilution was plated on six replicate plaques arranged over a triangular area occupying one of six equal sectors of the surface of the medium in a four-inch Petri dish, the base of the triangle being adjacent to the circumference of the plate and its apex pointing to the centre. In the later part of the work, up to five of the sectors were used for viable counts, the remaining portion of the plate being reserved for a check on the growing quality of the medium, by plating on this sector one small drop of 10^{-6} dilution of a suspension of standard turbidity of a 24-hour culture of *H. pertussis*. Fig. 1 shows the photograph of a typical lung-count plate.

In the earlier experiments, on unimmunized mice, the lung homogenates were diluted serially tenfold, the number of dilutions necessary to give countable colonies on the plaques, from mice given a particular infecting dose, being determined by preliminary experiments. This method would have required a prohibitive amount of labour in vaccine assays. In the later ones, the undiluted homogenate only was plated. The number of colonies in the plaques was counted, or if above 20 or so per plaque, was estimated by matching the plaque against a series of photographs of plaques the number of colonies in which had been determined by dilution and plating. It was considered that the error of this estimation was unlikely to exceed ±50 p.c. in plaques containing up to 500 colonies, a figure which was very seldom exceeded.

Expression of results of viable counts from the lungs. All calculations were performed on the logarithms of the viable counts, taken as the arithmetic means of counts on six plaques. Where no viable *H. pertussis* was recovered from the undiluted lung homogenate of an animal, the $\log_{10}$ value of -1 was arbitrarily assigned.

The term "apparent multiplication factor" is used to denote the difference between the logarithm of the viable infecting dose and the mean of the logarithms of the viable counts in the lungs of a group of mice. It is appreciated that not all organisms administered intranasally can be expected to reach the animals' lungs, and that the viable organisms recovered represent the balance between multiplication in the mouse, and destruction *in vivo* and during the processing. The figures are nevertheless considered to allow comparison between trends of fluctuation in the bacterial content of the lungs under varying conditions of dosage and immunization, since the technique of processing was kept constant.

RESULTS.

Viable counts in the lungs of unimmunized mice.

In a series of experiments, groups of mice were given, intranasally, total doses of 500 million, 50 million, 5 million, 500,000, 50,000 and 5,000 organisms

till a total of 50 counts on each dose level was obtained. Because the two highest dose levels were in the lethal range and, also, due to occasional contaminants, the size of the groups became irregular.

The lungs of most animals which received doses up to 5 million organisms appeared normal, macroscopically, although some were enlarged. Definite or gross pneumonic changes were present in some animals given similar, and in most of those given higher, doses.

Table 1 gives the means of the logs of the counts. In every experiment with the exception of one (No. 5) the viable count rose with increase in the infecting dose and reached a peak at 50 million organisms; it fell off at 500 million. In one experiment (No. 5) the highest viable count was obtained on the largest dose (500 million organisms). On the two largest doses, the viable counts were determined on survivors; during the week between dosage and the performance of the lung counts, the mean death rate of the animals receiving 500 million organisms was 49 p.c., and that of those given 50 million organisms, 28 p.c.

Contrasting with this regular trend were the fluctuations between the experiments. They appeared to be independent of, and were larger than might have been expected from, the variations in the percentage of viable organisms in the infecting doses. The fluctuations in the viable counts on the lung homogenates amounted to between 0·5 and 1 log unit in the groups receiving 50,000, 500,000 and 5 million organisms; between 1 and 2 log units in the groups given 5,000 and 500 million; and over 2 log units following the administration of 50 million bacteria.

The results were further analyzed (Table 2), using the apparent multiplication factors. These factors and their standard errors, which are also the standard errors of the means given in Table 1, are given in the upper part of the Table. In doses ranging up to and including 500,000 organisms, the number of organisms cultured from the lungs exceeded the viable infecting dose approximately tenfold. Following the infecting dose of 5 million organisms there was less multiplication, and there was practically none after 50 million bacteria. In mice surviving the infecting dose of 500 million organisms, the mean number of viable bacteria recovered from the lungs was about one-thirtieth of the number present in the infecting dose.

The upper part of Table 2 also shows the significance of the differences between the apparent multiplication factors in groups of mice receiving adjacent infecting doses within the same experiment. The significance of the difference was not calculated for groups on the lowest infecting dose, owing to the relatively large number of animals the lungs of which contained insufficient numbers of viable organisms to show up on culture, under the experimental conditions. The difference was statistically significant in 5 out of 15 pairs; in these, the

TABLE 1.

Viable counts in the lungs of mice 7 days after intranasal infection with *H. pertussis*.

No. of experiment	Per cent. viable organisms in infecting dose	Total number of *H. pertussis* in infecting dose													
		500 million			50 million			5 million		500,000		50,000		5,000	
		Per cent. deaths between inoculation and lung culture	No. of mice	Log mean viable count	Per cent. deaths between inoculation and lung culture	No. of mice	Log mean viable count	No. of mice	Log mean viable count	No. of mice	Log mean viable count	No. of mice	Log mean viable count	No. of mice	Log mean viable count
1	15	67	4	7·277	33	8	7·624	9	6·545	10	6·105	10(1)*	5·035	10(5)	3·835
2	8	55	8	6·806	40	11	7·087			19	5·510			4	4·390
3	10	21	9	6·363	15	14	6·942			18	5·642			5(2)	3·532
4	11	51	26	5·832	31	9	6·324								
5	10	30	3	6·119	12	8	5·398	8	5·832	3	5·314	8(4)	4·435	8(3)	3·751
6	9							15	6·497			9(1)	5·036	6	4·809
7	7							18	6·485			12	5·306	12(7)	3·361
8	9											11(1)	4·326	5	4·157

* The figures in brackets are the numbers of animals in the lungs of which there were insufficient viable organisms to show up in the cultures.

TABLE 2.

The Multiplication of *H. pertussis* in the lungs of mice: Analysis of the data of Table 1.

No. of experiment		Total infecting dose					
		500 million	50 million	5 million	500,000	50,000	5,000
1	Apparent multiplication factor	−0·596 (0·249) ←HS→	0·751 (0·305) ←NS→	0·672 (0·290) ←NS→	1·232 (0·126) ←NS→	1·162 (0·377)	0·962 (0·349)
2		−0·796 (0·200) ←NS→	0·485 (0·566) ←——NS——→		0·908 (0·351)		1·788 (0·237)
3		−1·336 (0·322) ←HS→	0·243 (0·360) ←——NS——→		0·943 (0·228)		0·833 (0·735)
4		−1·908 (0·257) ←S→	−0·416 (0·205)				
5		−1·580 (0·077) ←NS→	−1·301 (0·444) ←S→	0·133 (0·394) ←NS→	0·615 (1·295) ←NS→	0·736 (0·656)	1·052 (0·377)
6				0·844 (0·210) ←——NS——→		1·383 (0·329)	2·156 (0·297)
7				0·941 (0·161) ←——S——→		1·762 (0·200)	0·817 (0·307)
8						0·673 (0·363)	1·504 (0·496)
Mean of 50 mice		−1·502	0·012	0·734	0·968	1·170	1·192
Variance within experiments (and no. of d.f.)		1·188 (45)	1·743 (45)	0·695 (46)	1·514 (46)	1·430 (45)	1·171 (43)
Variance between expts. (and no. of d.f.)		2·956 (4)	5·784 (4)	1·293 (3)	0·389 (3)	2·209 (4)	1·751 (6)
Variance ratio (F)		2·488	3·318	1·860	3·892	1·545	1·495
Significance		NS	S	NS	NS	NS	NS

Apparent multiplication factor = difference between mean of log viable counts and log viable infecting dose.

HS = difference statistically highly significant ($P<0·01$).
S = difference statistically significant ($0·01<P<0·05$).
NS = difference statistically not significant ($P>0·05$).
d.f. = degrees of freedom.

The top part of the Table gives the apparent multiplication factors, their standard errors (in brackets) and the significance of the differences between the inoculum groups within the same experiment. (The significance was not calculated for the lowest inoculum group.)

The lower part of the Table gives the analysis of variances, calculated separately for each of the six inoculum groups.

higher apparent multiplication factor was associated with the lower infective dose, as it was also in 7 of the 10 pairs where the difference was not statistically significant.

The variance for each of the dose groups was analysed separately. The results are shown in the lower part of Table 2. On all dose levels, with the exception of one (500,000 organisms) the variance between the experiments was larger than the variance within them, but the difference was statistically significant in only one dose group. In one dose group (500,000 organisms), the variance within the experiments exceeded the variance between the experiments. This was due to the latter having a much lower value in this group than in the others (0·389 against a range of 1·293 to 5·784). The reason for this discrepancy is not known; in further tests, provided by the control groups in immunization experiments, it did not recur.

In the above analysis, the variance between the experiments includes errors in the estimation of the viable infecting dose. The variance within the experiments is made up from the following components: irregularities in the administration of the infecting dose; errors in the estimation of viable organisms in the lungs; and variability in response between mice.

Progress of viable counts in the lungs of unimmunized mice given 500 million organisms by the intranasal route.

This experiment was carried out in order to confirm the finding that, 7 days after inoculation, the lungs of mice surviving administration of this dose contained fewer viable organisms than were present in the infecting dose.

120 mice were given the infecting dose and groups sacrificed for evaluation of viable counts in the lungs at intervals after the intranasal dosage as follows: 1 hour, 1 day, 2 days, 3 days and 7 days.

The lungs appeared macroscopically normal 1 hour after dosage. Next day, considerable oedema and hyperaemia were present. Over the subsequent two days this changed into pneumonic consolidation which was still present on the 7th day. Many of the mice about to be sacrificed on the 2nd day and 3rd day were moribund.

TABLE 3.

Progress of viable counts in the lungs of mice given 500 million *H. pertussis* by the intranasal route.

Time since administration of infecting dose	Cumulative number of deaths	Number of mice sacrificed	Satisfactory lung culture obtained on	Apparent multiplication factor (and its standard error)
1 hour	0	15	15	−0·772 (0·296)
1 day	2	14	10	−0·248 (0·071)
2 days	37	15	7	−0·356 (0·114)
3 days	42	14	10	−0·553 (0·168)
7 days	52	10	8	−1·201 (0·543)

Apparent multiplication factor = (mean of log of viable counts) − (log viable infecting dose).

Table 3 shows the results. Most deaths occurred during the first three days. The severe illness of the animals sacrificed on the second and third days may have been the cause of the plate contaminants resulting in the unusually large proportion of unsuccessful cultures. In the first count, one hour after intranasal infection, approximately one-sixth of the estimated number of viable organisms administered was recovered from the lungs. During the next day, this number rose approximately threefold, thereafter to fall gradually to approximately one-twelfth of the infective dose on the 7th day. The apparent multiplication factor derived from the final count was of the same order as that observed in the previous experiment. None of the differences between the viable counts in this series was statistically significant.

Immunization with vaccine E75.

The immunizing effect of unheated and heated vaccine was compared in a total of 15 paired experiments. Intracerebral challenge was used in 5; lethal intranasal challenge (challenge dose, 500 million organisms) in 5; and low-dose intranasal challenge (500,000 organisms) with subsequent lung culture, in 5. The immunizing doses contained 2,000 million, 400 million, 80 million and 16 million organisms; the lowest dose was omitted in the case of the groups receiving the low-dose intranasal challenge. Fifteen mice were used per group. In the low-dose intranasal challenge groups, ten viable counts, selected at random from each group, were considered when calculating results. The 15 experiments were carried out over a period of five months; whenever possible, two paired tests, using two methods of challenge, were carried out concurrently.

The results are given in Table 4. In the lethal intranasal tests (experiments Nos. 1–5) very little overall protection was present, although in one (No. 3) the unheated vaccine appeared to be efficient. Altogether, in the control groups 25·3 p.c. (18 out of 71) of the animals survived; in the groups immunized with unheated vaccine, 29·3 p.c. (83 mice out of 283); and in the groups given heated vaccine, 30·4 p.c. (89 animals out of 293). In the groups challenged by the intracerebral route, the survival rates were: controls 4·2 p.c. (3 mice out of 71); unheated vaccine 8·8 p.c. (24 mice out of 273); heated vaccine 8·8 p.c. (24 mice out of 271). The survival rates in the immunized groups were higher than in the control groups; the difference was statistically just significant ($P=0·05$).

The boiling had no demonstrable effect on the overall protective power of the vaccine. It is to be noted, however, that following either method of challenge, the grading of the response according to the immunizing dose was more marked in the groups given unheated vaccine than in those given heated vaccine; in the latter, doses of 400 million and 80 million organisms protected better than the dose of 2,000 million organisms. Similar observations were also made in a subsequent experiment.

TABLE 4.

Immunization with Vaccine E75.

No. of expt.	Method of challenge	Vaccine		Not heated				Heated				Controls	Significance
		Dose (millions of organisms)		2000	400	80	16	2000	400	80	16		
1	Intranasal	Survivors/total		4/13	4/14	4/16	3/13	0/14	7/14	2/15	2/14	4/13	All receiving unheated vaccine (83/283) *vs* controls, NS
2	(lethal)			1/13	1/14	3/15	2/13	1/15	4/15	8/15	0/15	1/14	
3				13/15	13/15	3/14	4/14	6/15	6/14	12/15	6/15	4/15	All receiving heated vaccine (89/293) *vs* controls, NS
4				3/15	7/15	4/13	1/13	5/15	1/15	1/13	5/15	4/15	
5				4/15	2/14	6/12	1/15	6/15	6/15	9/15	2/14	5/14	
Total				25/73	27/72	20/70	11/68	18/74	24/73	32/73	15/73	18/71	
6	Intracerebral	Survivors/total		2/14	0/15	0/15	2/15	0/13	2/15	4/14	0/14	0/14	All receiving unheated vaccine (24/273) *vs* controls, S.
7				1/12	2/10	1/11	0/10	0/11	1/9	3/12	1/11	1/14	
8				3/15	1/15	0/15	1/14	1/14	2/14	1/15	1/13	0/15	All receiving heated vaccine (24/271) *vs* controls, S.
9				2/14	1/14	3/14	1/14	1/14	1/15	1/15	2/14	0/14	
10				0/14	2/13	0/15	2/14	2/13	1/15	0/15	0/15	2/14	
Total				8/69	6/67	4/70	6/67	4/65	7/68	9/71	4/67	3/71	
		Log viable infect. dose											
11	Intranasal	5·119	Log mean	5·543	5·653	5·998	—	6·063	5·828	6·145	—	6·369	Unheated *vs* heated S*.
12	(sub-lethal)	5·068	viable	5·491	5·411	5·711	—	5·843	6·070	6·184	—	5·841	
13		4·922	counts in	4·020	5·581	4·816	—	5·621	5·505	5·694	—	5·906	
14		4·512	lungs	4·562	5·107	5·571	—	5·392	5·616	6·069	—	5·646	
15		4·556		4·900	5·941	5·813	—	5·891	5·504	5·998	—	6·101	
Mean				4·903	5·539	5·614	—	5·762	5·705	6·018	—	5·973	

S = difference statistically significant ($P<0·05$).
NS = difference statistically not significant ($P>0·05$).
* Potency ratio = 47, with 5 per cent. confidence limits of 2·4 and 891.

Appreciable protection was demonstrated in the sublethal intranasal tests. Compared with the controls, the three dose levels of the unheated vaccine caused approximately tenfold, threefold and twofold reductions in the viable counts; the maximum reduction obtained with the heated vaccine was less than twofold. It may be noted that in only one group of mice, that given 2,000 million organisms of the unheated vaccine in experiment No. 13, was the mean viable count reduced to below the viable infecting dose. The results of these tests were analysed further by evaluating the relative potencies of the boiled and unboiled vaccines using the method described by Healy (1949). The calculations were made on the means given in Table 4, ignoring their standard errors; the reason for this is given below. The assay was found to be a valid one, the unboiled vaccine being 47 times more potent than the boiled, the 5 p.c. confidence limits being relative potencies of 2·4 and 891.

The boiling, therefore, destroyed most of the protective power of the vaccine, as shown by the sublethal intranasal test.

Immunization with vaccine K205.

Table 5 shows the results of paired tests on animals immunized with unheated and heated vaccine, and challenged by the lethal intranasal method (3 tests) and by the intracerebral method (2 tests). In the intranasal challenges, the animals were given 100 million organisms, and strains CN 1262 and GL 353 were used.

In the intranasal challenge, the total survival rate in three experiments in the groups receiving the unheated vaccine was 79 animals out of 191 (41·3 p.c.), and in those given heated vaccine 78 animals out of 191 (40·8 p.c.). In the control groups, 6 mice out of 46 (13 p.c.) survived. The protection was thus practically the same in the two immunized groups. It was, in both, highly significant ($P<0·01$) when compared with the controls.

Among the groups challenged by the intracerebral route, highly significant protection ($P<0·01$) was present in those receiving the two highest doses (2,000 million and 400 million organisms) of the unheated vaccine. On these two doses the survival rates were 25 animals out of 30 (83 p.c.) and 20 out of 30 (67 p.c.) respectively, as against 0 out of 30 in the control groups. Among the other immunized groups, the highest protective rate was 4 animals out of 30 (13 p.c.); the difference between this rate and that in the control groups is statistically not significant. These comparisons were made on the added results of two experiments. The difference between the total survival rates of the groups immunized with unheated vaccine (50 animals out of 120=41·7 p.c.) and heated vaccine (9 mice out of 120=7·5 p.c.) is statistically highly significant ($P<0·01$).

In this experiment, as in the previous one on vaccine E75, the response, using either method of challenge, to the unheated vaccine was graded according

TABLE 5.

Immunization with Vaccine K205.

No. of expt.	Method of challenge	Vaccine: Dose (millions of organisms)	Not heated: 2000	400	80	16	Heated: 2000	400	80	16	Controls	Significance
1	Intranasal (lethal)	Survivors/total	8/15	7/15	4/15	5/14	6/15	7/15	9/15	5/15	4/14	All receiving unheated vaccine (79/191) *vs* controls, HS
2			9/15	10/15	6/15	5/15	6/15	8/15	5/15	5/15	1/15	All receiving heated vaccine (78/191) *vs* controls, HS
3			8/18	8/18	5/18	4/18	6/18	10/17	7/18	4/18	1/17	
Total			25/48	25/48	15/48	14/47	18/48	25/47	21/48	14/48	6/48	
4	Intra-cerebral	Survivors/total	14/15	11/15	0/15	0/15	0/15	0/15	2/15	0/15	0/15	Groups given unheated vaccine, 2000 million (25/30) and 400 million (20/30) *vs* controls, HS Other groups *vs* controls, NS
5			11/15	9/15	4/15	1/15	0/15	3/15	2/15	2/15	0/15	
Total			25/30	20/30	4/30	1/30	0/30	3/30	4/30	2/30	0/30	All given unheated vaccine (50/120) *vs* all given heated vaccine (9/120), HS

HS = difference statistically highly significant ($P<0{\cdot}01$).
NS = difference statistically not significant ($P>0{\cdot}05$).

TABLE 6.

Comparison of Vaccines E75 and AIII.

No. of experiment	Method of challenge	Vaccine Dose (millions of organisms)		E75			AIII			Controls
				2000	400	80	4000	800	160	—
1	Intra-cerebral	Survivors/total		5/12	3/12	0/12	12/12	1/12	3/12	0/12
2 & 3 combined				—	—	—	27/30	7/30	1/28	3/30
4 to 8* combined				8/69	6/67	4/70	—	—	—	3/71
				13/81	9/79	4/82	39/42	8/42	4/40	6/113
	Intranasal (sub-lethal)	Log viable infecting dose								
9		4·446	Log mean viable counts	4·837	5·947	6·042	4·267	5·237	5·820	6·002
10		4·802		5·884	6·347	6·418	5·821	5·963	6·365	6·305

* Taken from experiments Nos. 6 to 10 in Table 4.
For discussion of relative potency, see text.

to the dose, whereas doses of 400 and 80 million organisms of the heated vaccine protected better than the dose of 2,000 million organisms.

In this experiment, the heating appeared to have no appreciable effect on the protective power of the vaccine against lethal intranasal challenge; it destroyed the protection the vaccine afforded against intracerebral challenge.

Comparison of vaccines E75 and AIII.

Table 6 shows the results of protection by these vaccines against intracerebral and intranasal (sublethal) challenges.

Against the intracerebral challenge, only one paired test could be carried out; in addition, two protection tests were performed on AIII and results of 5 experiments on unheated E75, already given in Table 4, have been included for comparison. On doses of 4,000, 800 and 160 million organisms of AIII, 93, 19 and 10 p.c. of the animals survived, as against 16, 11 and 5 p.c. of the animals given 2,000, 400 and 80 million organisms of E75. 5 p.c. of the controls survived. Doses of 800 million AIII and 2,000 million E75 gave similar protection, as did also doses of 180 million AIII and 400 million E75. AIII, therefore, appeared somewhat more potent than E75, but, since the series contained only one paired test, the relative potency could not be estimated accurately.

Two paired tests were made using the small-dose intranasal challenge. The means of the logs of the viable counts in the groups of mice were analysed by the method of Healy (1949). The assay proved a valid one. On the doses used, AIII was found to be 4 times as potent as E75, the upper 5 p.c. limit of the potency ratio was over 6·3 and the lower 5 p.c. limit under 0·15. Since the immunizing doses of AIII were twice those of E75, the actual potency ratio was 2.

Both methods thus indicated that AIII was slightly better than E75.

Interference immunity.

Evans and Perkins (1954a, 1954b) demonstrated specific immunity in mice against intracerebral infection within a few hours after intraperitoneal injection of pertussis vaccine. They considered the phenomenon was due to interference.

In a number of experiments, the effect of vaccine E75 was examined for its ability to produce the interference effect. The vaccine—2,000 million organisms—was injected intraperitoneally; challenge followed 5 hours later. In three experiments, the challenge was by the intracerebral route; in two of these unheated vaccine was used, and in one, heated vaccine. In a further experiment, the mice were given unheated vaccine and challenged with a sublethal dose (500,000 organisms) by the intranasal route.

The results are shown in Table 7, which also includes, for comparison, some figures extracted from the results of experiments Nos. 6–10 in Table 4.

TABLE 7.

Interference immunity following the intraperitoneal injection of Vaccine E75 in doses of 2000 million organisms.

Total no. of experiments	Method of challenge	Immunizing agent	Interval between immunization and challenge	Survivors/total		Significance of difference (immunized/controls)	
				On the 9th day	On the 15th day	On the 9th day	On the 15th day
2	Intra-cerebral	Vaccine, not heated	5 hours	20/68	7/68	S	NS
		Nil (controls)		8/69	3/69		
1	Intra-cerebral	Vaccine, heated	5 hours	10/54	8/54	NS	NS
		Nil (controls)		13/59	11/59		
5*	Intra-cerebral	Vaccine, not heated	14 days	13/69	8/69	NS	NS
		Nil (controls)		7/71	3/71		
5*	Intra-cerebral	Vaccine, heated	14 days	8/65	4/65	NS	NS
		Nil (controls)		7/71	3/71		

				No. of mice in group	Log viable infecting dose	Mean of log viable counts (and its standard error)	
1	Intranasal (low dose)	Vaccine, not heated	5 hours	23	4·843	6·250(0·055)	HS
		Nil (controls)		23	4·843	6·501(0·065)	

* Taken from experiments 6-10 in Table 4.
HS = difference statistically highly significant ($P<0·01$).
S = difference statistically significant ($0·01<P<0·05$).
NS = difference statistically not significant ($P>0·05$).

Among the groups challenged by the intracerebral route, the survival rate of the animals given unheated vaccine was slightly over twice that in the corresponding control groups. This difference was statistically significant ($0{\cdot}01<P<0{\cdot}05$) in the group challenged 5 hours after immunization, the survival rates being taken at 9 days after challenge. In the same group at 14 days after the challenge, and in the groups challenged 14 days after immunization and read either at 9 or at 14 days after challenge, the proportions of survivors in the immunized groups were about twice as high as in the control groups, but the difference was statistically not significant ($P>0{\cdot}05$). The mice given heated vaccine and challenged by the intracerebral route showed very little protection whether challenged 5 hours or 14 days after immunization, and irrespective of how long after challenge the results were read. The interference effect was thus present in mice given unheated, but not in those given heated, vaccine.

The interference effect was clearly demonstrated in the mice immunized with unheated vaccine and challenged by the intranasal technique. The count in the control group exceeded the count in the immunized group 1·8-fold, the difference being highly significant ($P<0{\cdot}01$). It is to be noted that the counts within these two groups were highly uniform as shown by the standard errors of their means (0·055 and 0·065, respectively). In other experiments (e.g., in those described in Tables 2 and 5), the standard errors of the means of counts on the lungs of mice receiving the same infecting dose were much larger. The mice used in the interference experiment weighed 15–18 gm. at the time of challenge, whereas the others were heavier (18–22 gm.) when challenged.

DISCUSSION.

The work presented consists of an examination of the multiplication of *H. pertussis* in the lungs of normal and of actively immunized mice. The viable counts in the lungs were made one week after the administration of the infecting dose by intranasal instillation. In preliminary experiments, the highest counts were obtained at that time, a result in conformity with the reports of Proom (1947) and Cooper (1952).

The total doses used in the experiments on normal animals ranged from 5,000 to 500 million organisms per mouse. The doses were standardized according to opacity and contained approximately 10 p.c. viable organisms. In one trial, viable counts were made on the lungs of mice one hour after intranasal dosage; one-sixth of the estimated viable infecting dose was recovered. This fall may have been due to not all viable organisms in the infecting dose reaching the lungs. The assumption that they did so reach the lungs was nevertheless made for the sake of convenience in presenting the results, and it was also assumed for the same reason, that the viable counts in the lung homogenates were a true estimate of the number of living *H. pertussis* in the lungs.

During the experimental period—one week—the number of viable *H. pertussis* in the lungs rose approximately tenfold, on infecting doses varying from 5,000 to 500,000 bacteria. When 5 million organisms were administered, the multiplication rate was lower; on 50 million there was very little multiplication, and after a dose of 500 million organisms, less than one-tenth of the estimated number of viable infective organisms was recovered from the lungs. In 4 experiments out of 5, fewer viable bacteria were recovered from the lungs of mice given 500 million organisms than from those dosed with 50 million. This might have been due to survival of mice which had not taken the dose well. However, viable counts on the lungs of mice given the highest dose (500 million organisms) and sacrificed in groups over one week after dosage, showed a rise during the first 24 hours, then a gradual fall to the 7th day; during the first half of the week the mice were ill or moribund when about to be sacrificed. By contrast, viable counts made 1, 3 and 6 days after doses of 50,000, 500,000 and 5 million organisms, showed steady rises; these experiments have not been described. It was also noted that, while doses of 500,000 organisms or less caused no visible changes in the lungs, gross pneumonia was present in some animals given 5 million organisms, and in practically all on higher doses.

These results are explained tentatively as follows. Large doses (500 million bacteria) caused the host to respond in a way which led to the destruction of comparatively large numbers of bacteria; the products of this destruction affected, and in some cases killed, the host. If the host survived the experimental period its lungs contained fewer living organisms at the end than at the beginning. The pneumonic changes were caused by the living organisms and the products of their destruction. Lower doses were insufficient to call forth the host response leading to wholesale destruction of the organisms; no gross pulmonary changes were present; during the experimental period, the balance of bacterial multiplication and destruction by the host favoured the invader, leading to a steady rise in numbers. After one week the gradual building up of the host's resistance would tend to eliminate the organisms.

The interference phenomenon (Evans and Perkins, 1954a and 1954b) may have been a further factor in bringing about the effect of large infecting doses (500 million organisms) since they contained large numbers of dead organisms (usually about 90 p.c.); these may have interfered with the multiplication of the living bacteria. Interference immunity, manifested by the reduction in bacterial multiplication in the lungs of mice given pertussis vaccine intraperitoneally a few hours before intranasal inoculation, could indeed be demonstrated against small infecting doses (500,000 organisms). North (1955), however, could find no protection against death in mice given pertussis vaccine by the intranasal route and challenged by the same route with large doses of *H. pertussis* a few hours later; the number of viable organisms in the lungs of mice in this group was not determined.

Thus it appeared that the death of mice infected with large doses might be prevented either by checking the multiplication of the organisms or by neutralizing the harmful effects caused by their destruction. If that were so, immunity against lethal intranasal infection would depend on two factors; against sublethal infection on one, since only reduction in the intrapulmonary multiplication of the bacteria would be tested for. The immunization experiments were performed to find out whether the lethal and sublethal intranasal methods measured the same property of vaccines; the intracerebral method was also included.

The sublethal intranasal challenge dose for immunization experiments, 500,000 organisms, was chosen because it was in the range where intrapulmonary multiplication was maximal, and high enough to give positive lung cultures, under the experimental conditions, in practically all unimmunized animals.

In the lungs of successfully immunized mice challenged by the sublethal intranasal method, the rate of bacterial multiplication was significantly lower than in unimmunized controls, a result in conformity with the work of North *et al.*, quoted above. Multiplication of the organisms was seldom completely prevented; the degree of depression of the multiplication was graded according to the immunizing dose given. The immunizing power of the vaccine against this method of challenge was greatly impaired by boiling.

The results of the experiment on vaccine K205 were in conformity with the findings of Standfast (1953), who observed that boiling destroyed the protective power of pertussis vaccine against intracerebral, but not against lethal intranasal challenge. Vaccine E75 protected only very slightly against intracerebral challenge; this effect was not destroyed by boiling. In both intracerebral and lethal intranasal tests on vaccines E75 and K205, protection by unheated vaccine was graded better according to the immunizing dose than with heated vaccine; the survival rates on the highest dose (2,000 million organisms) of the latter were consistently lower than on lower doses. The reason for this phenomenon is not clear; it is possible that the boiling unmasked a hypersensitizing effect which became evident when large doses were given, and affected the animals unfavourably after challenge. A comparison of vaccines AIII and E75 showed AIII slightly superior by both intracerebral and sublethal intranasal techniques; less vaccine was required for the demonstration of immunity by the latter method than by the former.

On the whole, the results of the low-dose intranasal challenge of immunized mice resembled the results of the intracerebral challenge more than the results of the lethal intranasal challenge. That the lethal and sublethal intranasal challenges would give different results in immunized animals was not unexpected, since, as already pointed out, the administration of corresponding challenge doses to unimmunized animals resulted in differing rates of multiplication, and pathological changes, in the lungs.

In the experiments involving sublethal intranasal challenge described above, and in a number not described, it was observed that fluctuations in viable counts between experiments were larger than mouse to mouse variations within the one test; moreover, in some immunization experiments the variations within groups showing protection were much greater than in unimmunized or unprotected groups—a result not unexpected, since immunization introduces an additional variable. Owing to these two factors relative potencies of vaccines were calculated from mean viable counts of groups of mice, ignoring the degree of variation within the groups. This procedure is justified if the relative potencies so calculated are reproducible; that was so in our limited experience where both standard and unknown were at least moderately potent. More data will be required for proof.

SUMMARY.

Viable counts on lungs of mice, 1 week after intranasal instillation of suspensions of virulent *H. pertussis* showed that, in the sublethal range, larger infecting doses were followed by higher counts. In the lethal range, the viable counts reached a maximum; further increase of the infecting dose merely increased the rate of killing. Gross changes were present in the lungs of mice given doses in the lethal range, but not in animals given smaller doses.

In active immunization tests the intracerebral and sublethal intranasal tests were similar in that the protective agent was destroyed by boiling the vaccines; a smaller degree of immunity could be detected by the latter test. The agent protecting against lethal intranasal challenge was more stable to heat.

Interference immunity could be demonstrated against the sublethal intranasal challenge.

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REFERENCES.

Anderson, G. and North, E. A. (1943): Austral. J. exp. Biol., 21, p. 1.
Burnet, F. M. and Timmins, C. (1937): Brit. J. exp. Path., 18, p. 83.
Cohen, S. M. and Wheeler, M. W. (1946): Amer. J. Pub. Health, 36, p. 371.
Cooper, G. N. (1952): J. Path. Bact., 64, p. 65.
Evans, D. G. and Perkins, F. T. (1954a): Brit. J. exp. Path., 35, p. 322.
Evans, D. G. and Perkins, F. T. (1954b): *Ibid.*, 35, p. 603.
Fisher, S. (1950): Austral. J. exp. Biol., 28, p. 509.
Fisher, S. (1953): Riassunti delle Communicazioni, 6th International Congress of Microbiology, Paper No. 810.
Healy, M. J. R. (1949): Biometrics, 5, p. 330.
Kendrick, P. L., Eldering, G., Dixon, M. K. and Misner, J. (1947): Amer. J. Pub. Health, 37, p. 803.

Keogh, E. V. and North, E. A. (1948): Austral. J. exp. Biol., 26, p. 315.
Lacey, B. W. (1951): J. gen. Microbiol., 5, p. vi.
Lacey, B. W. (1954): J. Hyg., 52, p. 273.
Miles, A. A. and Misra, S. S. (1938): *Ibid.*, 38, p. 732.
Nicholson, D. E. and Turner, G. C. (1954): J. gen. Microbiol., 11, p. 91.
North, E. A. (1946): Austral. J. exp. Biol., 24, p. 253.
North, E. A. (1955): Unpublished observations.
North, E. A. and Anderson, G. (1941): Med. J. Austral., I, p. 754.
North, E. A. and Anderson, G. (1942): *Ibid.*, II, p. 228.
North, E. A., Anderson, G. and Graydon, J. J. (1941): *Ibid.*, II, p. 589.
North, E. A., Keogh, E. V., Anderson, G. and Williams, S. W. (1939): Austral. J. exp. Biol., 17, p. 275.
Proom, H. (1947): J. Path. Bact., 59, p. 165.
Standfast, A. F. B. (1953): Unpublished observations.
Warburton, M. F. and Fisher, S. (1951): Austral. J. exp. Biol., 29, p. 265.

ORNITHOSIS IN THE PETREL, *PUFFINUS TENUIROSTRIS* (TEMMINCK)

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The short-tailed shearwater (*Puffinus tenuirostris*), popularly known in south-eastern Australia as the mutton bird, breeds on the islands around Tasmania and the south-eastern Australian mainland from Gabo Island in the east to St. Francis Island in the west. Commercial exploitation takes place on the small islands in the Furneaux Group in eastern Bass Strait. Interest in this species was evoked not only because of its habits, but also because of its economic importance. The carcases of fledglings are sold as a table delicacy and various by-products such as feathers, body fat and proventricular oil are also marketed. An intensive study of its ecology was started in 1947 by Dr. D. L. Serventy, of the Wildlife Survey Section of the Commonwealth Scientific and Industrial Research Organization, and is still in progress. The investigations leading to the results reported in this paper were part of this programme.

Materials and Methods.

Animal inoculations. Mice bred in the Australian National University Animal Breeding Establishment in Canberra, and also at the Institute of Medical and Veterinary Science, Adelaide, were used. A.N.U. mice were inoculated when 6–8 weeks old, and I.M.V.S. mice when 3 weeks old. Intranasal inoculation was carried out under anaesthesia using 0·05 ml. volumes of inoculum. Intracerebral inoculations were made with 0·03 ml. volumes and intraperitoneal with 0·5 ml. volumes.

Inocula which were prepared from organs of fledgling or adult breeding birds were emulsified in isotonic borate buffered saline containing 0·5 p.c. gelatine. Spleen, lung, kidney and liver were used. Smears were made from the organs used for virus isolation experiments and stained by Giemsa's and Gram's methods.

For egg inoculation experiments 6–8 day old developing chick embryos were used. Inoculation was *via* the yolk sac.

Mutton birds used in virus isolation experiments. Twelve live fledgling birds were sent to the I.M.V.S., Adelaide, during the commercial "birding" season of 1953.

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In November, 1954, during the period of southern migration (*vide infra*) carcases of adult birds were collected from a Sydney beach. The organs of some were preserved in 50 p.c. glycerol-saline; others were used immediately for experimental animal inoculation.

Serological tests.

The complement fixation test (CFT) and complement fixation inhibition test (CFI) were carried out as follows:—

In studying the virus strain isolated in November, 1954, from a migrating bird, and in testing sera collected in 1955, a heat-killed antigen prepared from lungs of mice infected with this virus was used. The method of preparation was that described by Bedson (1937). On this occasion the CFT was performed following the single drop method described by Donnelley (1951). Serial 2-fold dilutions of serum were tested in the presence of 3 HD_{50} of complement in calcium magnesium saline (Mayer *et al.*, 1946) and a 1 in 4 dilution of standard antigen suspension. The indicator system—3 p.c. sheep's R.B.C.'s sensitized with 8 HD_{50} of haemolysin—was added after fixation overnight at 4° C. The result was read after incubation in a water-bath at 37° C. for one hour.

For mutton bird sera collected in 1955 the following technique was used for the CFI. To serial 2-fold dilutions of serum in calcium magnesium saline, beginning at 1 in 1, 2½ units of antigen were added. This mixture was placed overnight at 4° C. After holding for one hour at room temperature, 3 HD_{50} of complement and two units of positive serum were added. The mixture was incubated for one hour in a water-bath at 37° C. before the addition of sensitized cells. The result was read after returning the test to the water-bath for a further hour. Positive human serum "Bernard," supplied by the I.M.V.S., was used. The highest dilution of antigen or serum giving a positive reading in the CFT was taken as a unit for the CFI test.

The mutton bird sera collected in 1953 and 1954 were tested with an antigen prepared from virus of enzootic abortion of ewes, using the method described by Dane (1955). The CFT and CFI techniques used were described in detail by Beech and Miles (1953).

Collection of sera. Mutton birds were bled from the brachial vein. Sera were separated soon after collection and stored in a refrigerator before being despatched in a thermos flask to the laboratory where they were stored at −15° C. They were inactivated at 56° C. for 30 minutes before use.

Sensitivity to antibiotics in ovo. The ability of various drugs to delay the death of chick embryos inoculated with virus beyond the time when similarly inoculated control eggs had died, was tested using the method described by Meiklejohn *et al.* (1946).

Tissue culture techniques. A modification of the technique used by Weiss and Huang (1954) for growing feline pneumonitis virus in the avascular membrane (AVM) of the yolk sac of chick embryos was employed in studying the virus isolated from a mutton bird in 1953. The AVM was removed from a 3-day-old chick embryo and cut into pieces approximately 2 mm. square. These pieces of tissue were embedded in a small plasma clot on the middle of a glass slide 75 mm. by 9 mm. Each slide was placed clot downwards in a test tube 10 cm. by 1·3 cm. containing 1 ml. of medium (75 p.c. Hank's balanced salt solution and 25 p.c. horse serum). The test tube was fitted with a rubber stopper and placed in a rack horizontally; the quantity of medium was then sufficient to fill the space between the lower surface of the slide and the test tube. Cultures were incubated at 37° C. for 4 days and then one drop of virus suspension was added. After further incubation for 48 hours the slides were removed from the test tubes, fixed in methyl alcohol, stained by Giemsa's method, and examined under the microscope.

RESULTS

Isolation of a virus from a fledgling in 1953.

In May, 1953, 12 juvenile mutton birds were sent from the Bass Strait islands to Adelaide by air. On arrival a blood sample was taken from each bird and tested for anti-bodies to psittacosis group antigen using the complement fixation test (CFT) and also the complement fixation inhibition test (CFI). In no case was the CFT positive, but 5 of the 12 birds had titres of 1 in 5 or greater in the CFI, the highest titre being 1 in 40. Nineteen days after arrival in Adelaide one of the birds died. Post-mortem examination revealed gross muscle wasting and absence of the usual subcutaneous and perivisceral fat, but no other abnormality. The spleen was pale and small. A 10 p.c. suspension of the spleen was inoculated *via* the yolk sac into four 6-day-old eggs. These eggs died after 5 days and smears of the yolk sac showed numerous basophilic bodies about 500 mμ diameter when stained by Giemsa's method. Blood agar cultures of the yolk sac were sterile. The virus was readily passed in the yolk sac and in later passages killed the embryo after 3 days. Inoculated intracerebrally into 3-week-old mice it caused a rapidly fatal illness. A 10 p.c. suspension of infected yolk sac when titrated by intracerebral inoculation of groups of 6 mice for each log dilution had a titre of $10^{6.5}$ ID_{50} per ml. The same suspension had a titre of $10^{4.2}$ when given intranasally, but failed to produce any obvious infection when inoculated intraperitoneally. The virus was sensitive *in ovo* to penicillin (250 units), but not to streptomycin (1 mg.) or to sulphacetamide (20 mg.).

Proof that the agent isolated was a virus of the psittacosis group was obtained by inoculation of tissue culture and subsequent microscopic examination. Cultures of avascular membrane (AVM) from 3-day-old chick embryos were inoculated with a yolk sac suspension of virus. The AVM cells were examined 48 hours later after staining by Giemsa's method. Numerous cells contained basophilic inclusion bodies made up of minute elementary bodies. These inclusions were indistinguishable from those produced by known strains of psittacosis virus in similar tissue culture cells after a similar length of time.

The ease with which the virus was isolated in the first place suggests that it was present in the spleen in higher titre than is usual in healthy birds with a latent infection. What part the virus infection played in causing the bird's death is not clear. Malnutrition had probably been an important factor. Rapid virus multiplication may have been a terminal event because no pathological changes characteristic of fatal psittacosis infection were found.

Isolation of virus from a migrating adult bird in 1954.

A periodic mortality has been observed repeatedly among mutton birds migrating from their winter feeding grounds in the northern hemisphere to Bass Strait. From October to January numbers of carcases are washed ashore on the eastern seaboard of Australia. Exceptional mortality was recorded in

1954 and efforts were made during November to investigate the cause. During an inspection of the Boat Harbour Beach at Cronulla, approximately 2,000 carcases were counted on the four-mile stretch of shore, but only a few were sufficiently fresh for pathological study. Five carcases were dissected on the beach, and the organs (lung, liver, spleen, kidney and heart) were preserved in 50 p.c. glycerol-saline, and then stored for a few days in the deep freeze before being emulsified and used for mouse inoculations. Four other fairly fresh carcases were dissected in the laboratory, and organs emulsified in gelatine saline were used immediately for mouse inoculation.

Culture of these organs on nutrient agar, blood agar, and in serum broth, revealed a growth of Gram positive cocci and coliform organisms. The same organisms were seen in stained smears. However, they did not exhibit any pathogenic effect on mice when injected intracerebrally, intranasally or intraperitoneally.

The degree of infestation of the dead birds with the nematodes[3] *Stegophorus stellae polaris* (Parona, 1901) and *Seuratia shipleyi* (Stossich, 1900), and with a cestode—possibly *Tetrabothrium* sp., did not differ from that found in healthy birds.

One out of nine of the suspensions prepared from the organs of dissected carcases killed inoculated mice. The following findings were recorded on the autopsy of this bird: The carcase was fresh and well preserved. A serous exudate with an admixture of blood was observed coming from the nostrils. The lungs were haemorrhagic, and the abdominal air sacs were filled with a bloody exudate. The spleen was of normal size, but the liver was slightly swollen, and the cloaca filled with excreta.

All mice which were inoculated intracerebrally and intranasally died. The intraperitoneal route of infection was less lethal, with prolonged survival time and some recoveries.

On post-mortem examination all infected mice had extensive consolidation of their lungs. Inoculum prepared from infected lungs suspended in normal saline was passaged seven times by intranasal inoculation of mice. The survival times of infected mice were reduced on passage, the average survival times of successive passages being 9·0, 5·0, 5·4, 5·2, 5·0, and 3·0 days.

The infectious agent could not be grown by aerobic or anaerobic culture of suspensions of lung from infected mice. It was cultivated in eggs inoculated *via* the yolk sac. From eggs in which the embryos died on the fifth day after inoculation yolks and portions of yolk sacs were harvested, emulsified and used for intranasal inoculation of mice. All ten inoculated animals died in 5–12 days showing characteristic pathological changes in the lungs.

[3]Identifications of helminths made by S. Prudhoe of the British Museum of Natural History.

Identification of the agent as an ornithosis virus was accomplished by testing a heated antigen prepared from the lungs of mice which died, against a known human psittacosis anti-serum. It was also used in testing the sera of nine mice which had been inoculated with the 1954 strain of virus, and survived (see Table 1).

TABLE 1.

CF test with heat killed antigen prepared from 1954 strain, with sera of mice surviving the infection, and known human psittacosis anti-serum.

Serum	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320
normal mice	−	−	−	−	−	−	−
known human psittacosis anti-serum	+	+	+	+	+	+	−
2 mice	+	+	+	+	+	+	±
1 mouse	+	+	+	+	+	+	−
1 mouse	+	+	+	+	+	−	−
1 mouse	+	+	+	−	−	−	−
4 mice	−	−	−	−	−	−	−

Three of the mouse sera had titres of 1 in 160, one had a titre of 1 in 80, one a titre of 1 in 20 and the remaining four were negative at 1 in 5.

Examination of sera.

One hundred and seventy-five sera collected in March, 1954, during the early period of the commercial "birding season," were taken from the birds on Little Green Island. They represented a random sampling of different topographical regions in a rookery covering about 600 acres, situated in the north-west corner of the island. Only 10 serum samples were from adult birds, as they rarely remain in their burrows during the daytime—the remaining 165 were from fledglings.

All sera were tested for complement fixing antibodies, but none gave positive results. However, 34 of the 165 sera from juveniles birds had titres of 1 in 5 or greater in the complement fixation inhibition test. Sera from 6 birds gave titres of 1 in 40 or greater. Half of the ten adult sera tested had titres of 1 in 5 or greater (see Table 2).

TABLE 2.

Results of complement fixation inhibition tests on sera from mutton birds.

March 1954	<5	±5	5	10	20	≧40*	No. tested	No. positive	Percentage positive
Juveniles	124	7	16	9	3	6	165	34	21
Adults	4	1	4	0	1	0	10	5	50
January 1955	<4	4	8*				No. tested	No. positive	Percentage positive
Adults	45	7	8				60	8	13

* Reciprocal of serum titre.

Post-mortem examination of 44 of these birds failed to show any of the pathological changes associated with ornithosis and enlarged spleens were not found in the birds with demonstrable antibody. The spleen of juveniles has always been found to be considerably larger than that of adults. The average volume of the spleens of adult birds was 0·12 ml. and of juveniles 0·32 ml.

There was no correlation between a positive ornithosis titre and symptoms of "limey bird" disease. ("Limey bird" disease is the popular name for a condition prevalent among nestlings and characterized by diarrhoea.) Nor did measurements of wing length or culmen indicate any retarded development in chicks with positive titres. Weight gave no indication of a fledgling's condition as it varied with the lapse of time since the last feed. There was no indication that birds with serological evidence of ornithosis infection came from any particular type of terrain.

Sixty sera from adult birds were collected in January, 1955, during the late stage of brooding, on Little Green Island (40 sera), and from the neighbouring Great Dog Island (20 sera). On both islands efforts were made to obtain a random sampling of the rookeries. From the 60 sera tested, eight (13·0 p.c.) gave positive results by the CFI test at a dilution of 1 in 8, and 7 (11·5 p.c.) at 1 in 4 (see Table 2).

DISCUSSION.

The results given in this report show that mutton birds (*Puffinus tenuirostris*) are commonly infected with ornithosis virus. The birds must frequently become infected as fledglings because antibodies were demonstrated in the blood of 21 p.c. of juveniles examined in 1954. These juvenile birds were between 10 and 11 weeks old, an age at which detectable maternal antibody would have been lost. Isolation of virus in 1953 from a juvenile bird provides further evidence that infection can take place early in life. The nesting habits of the mutton birds are such that contact between juvenile birds does not normally occur until they are approximately 16 weeks old. At this age they start making short nocturnal excursions from their nesting burrows to sea shortly before their departure early in May. With this degree of isolation from all other birds but their parents, it seems likely that the parents are the source of infection. Thus a large number of breeding adult birds must at some time be actively shedding virus.

Under favourable conditions infection with the virus does not appear to cause any significant pathological changes in young or adult birds. There are no reports of fledglings or their parents dying with signs attributable to ornithosis, nor has any large scale mortality being observed among first year birds or adults on their northern migratory flight.

The position may be different with adults on their return flight from the northern hemisphere. The periodic mortality among them has been correlated

with the local absence of shoals of southern bluefin tuna (*Thunnus maccoyii*). These fish rely mainly on the same source of food as the mutton bird (D. L. Serventy, 1949). The food supply along the flight route of the birds off the eastern shore of Australia is of importance but we must also consider the possible rôle of ornithosis. It has been shown repeatedly that different species of bird may be carriers of ornithosis without showing any clinical signs of the disease. Only when their condition is weakened by such factors as overcrowding, malnutrition, exposure to abnormal environmental temperatures or the stress of breeding is the virus provoked to activity. Agents capable of activating different latent infections through weakening of the physiological resistance of the host have been discussed by Dubos (1955). A good example of the importance of the nutritional factor in inducing the disease is provided by the appearance of ornithosis among a group of experimental pigeons kept on a thiamine-deficient diet (Pinkerton and Swank, 1940). In this connection it is interesting to note that the juvenile mutton bird from which virus was so readily isolated in 1953 had been fed on an unsuitable diet.

The proportion of cases in which virus was isolated from migrating birds is not a true indication of the prevalence of the disease, because almost all the carcases used for virus isolation experiments were in an advanced stage of putrefaction. The ease with which virus was isolated from the organs of one migrating bird in 1954 when considered with the post-mortem findings strongly suggest that this bird had died of ornithosis and was not merely a carrier. It is hoped that further work on sick or recently dead birds will give a more accurate picture.

The southern migration appears to fall in to two phases. The heaviest concentration of birds passes the eastern seaboard of Australia between the third week in September and the end of October. These are the mature adults, which will breed during the current season. Numbers fall off from November to December, when the birds mostly comprise the immature adults which have not reached the breeding stage. Mortality among migratory birds is highest during this latter period, and we think therefore that ornithosis may have a more adverse effect on the young adults than on the more mature birds, and that it may be a contributing factor in the natural control of the mutton bird population.

Because ornithosis viruses are known at times to cause human infection, the possibility of mutton birds being a source of such infection in man was considered. It is known that another member of the Petrel family, the Fulmar (*Fulmaris glacialis*), was the source of infection for Faroe Islanders who used to salt the birds down for winter food (Haagen and Mauer, 1938). Before the taking of young fulmars was prohibited yearly outbreaks of a severe pneumonia, caused by ornithosis virus, used to occur. No cases of ornithosis-like disease in man have yet been reported from the Bass Strait islands. However, the virus

is pathogenic for man, as one of us (R.M.) had a moderately severe attack of ornithosis, with signs and symptoms of pneumonitis, whilst working with the 1954 strain of virus.

SUMMARY.

Two strains of ornithosis virus have been isolated from the mutton bird (*Puffinus tenuirostris*), in 1953 from a juvenile, and in 1954 from an adult bird.

Serological tests indicate that ornithosis infection is both common and widespread in mutton birds.

Juvenile birds are thought to acquire the infection from their parents early in life.

Evidence is presented that ornithosis may play some part in causing the mortality observed among migrating mutton birds.

No association could be found between ornithosis infection and the so-called "Limey bird" disease of juvenile mutton birds.

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REFERENCES.

Bedson, S. P. (1937): Lancet, 2, p. 1477.
Beech, M. D. and Miles, J. A. R. (1953): Austral. J. exp. Biol., 31, p. 473.
Dane, D. S. (1955): Med. J. Austral., 1, p. 382.
Donnelley, M. (1951): Austral. J. exp. Biol., 29, p. 137.
Dubos, R. J. (1955): Bull. N.Y. Acad. Med., 31, p. 5.
Haagen, E. and Mauer, G. (1938): Zbl. Bakt., 143, p. 81.
Mayer, M. M., Osler, A. G., Bier, O. G. and Heidelberger, M. (1946): J. exp. Med., 84, p. 535.
Meiklejohn, G., Wagner, J. C. and Beveridge, G. W. (1946): J. Immunol., 54, p. 1.
Pinkerton, H. and Swank, R. L. (1940): Proc. Soc. exp. Biol. Med., 45, p. 70.
Serventy, D. L. (1951): Proc. 7th Pacific Science Congress, N.Z., p. 394.
Weiss, E. and Huang, J. S. (1954): J. infect. Dis., 94, p. 107.

STUDIES ON INTERMEDIARY IRON METABOLISM.

VII. THE EFFECT OF SPLENECTOMY ON THE ABSORPTION AND STORAGE OF IRON IN EXPERIMENTAL ANAEMIA

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It was shown in another paper (Kaldor, 1954c) that the amount of storage iron was increased in the liver and spleen of rats recovered from anaemia induced by prolonged treatment with phenylhydrazine. The increased storage could be attributed to increased absorption of iron from food during the anaemia phase.

The anaemia induced by phenylhydrazine is due to haemolysis and is not associated with iron deficiency. Iron liberated from the destroyed erythrocytes is conserved in the body and is readily available for haemoglobin synthesis. Therefore, in this type of anaemia, increased absorption is not the result of physiological demand for iron. A better understanding of the mechanism responsible for the absorption of excess iron under these conditions is desirable not only for theoretical reasons but because a similar mechanism is believed to be responsible for the haemosiderosis of individuals who suffer from anaemia over a long period (Aufderheide, Horns, Goldish, 1953).

The following observations, made in the earlier experiments (Kaldor, 1954c) suggest that the spleen may have more than a passive rôle in the development of iron excess.

(1) In normal rats the liver stores more iron than the spleen. On the other hand, in rats treated with phenylhydrazine the capacity of the spleen for storing excess iron was much greater than the capacity of the liver.

(2) The weight of the spleen was increased in rats which absorbed and stored excess iron as a result of treatment with phenylhydrazine, but the degree of hypertrophy was greater in rats which received supplementary iron in their diet than in those rats which were maintained on a normal diet during treatment.

In the present experiments, splenectomized rats and intact rats were rendered chronically anaemic by injections of phenylhydrazine. As a result of this treatment, both group of rats absorbed increased amounts of iron from food. In the absence of the spleen it was found that the liver assumed the storage

[1]This work was undertaken during tenure of a Fellowship from the National Health and Medical Research Council.

function for iron of this organ. This was evident from the finding that the iron content of the liver of splenectomized rats was equal to the iron content of the liver and spleen combined in intact rats.

Materials and Methods.

General plan of the experiment.

Intact and splenectomized female rats, 10 to 12 months old and 220±25 gm. body weight, were grouped and treated as shown in the following scheme.

Intact Rats			Splenectomized Rats		
Group designation	No. of rats	Treatment	Group designation	No. of rats	Treatment
1A	10	None	2A	11	None
1B	10	Anaemia maintained by repeated injections with phenylhydrazine over a period of four months.	2B	11	Same as Group 1B
1C	10	Anaemia maintained by repeated injections with phenylhydrazine over a period of four months. Diet supplemented with iron during the same period.	2C	11	Same as Group 1C

It was ascertained that the previously anaemic rats had completely recovered from the anaemia one month after discontinuing injections. All rats were sacrificed at this time and the blood and tissues were collected for iron analysis.

Splenectomy. Splenectomy was performed on the rats of the groups 2A, 2B and 2C one month before the beginning of the experiment. A week before the operation, each rat was injected subcutaneously with 0·1 ml. of a 5 p.c. solution of neoarsphenamine as a protection against *Bartonella muris*. This organism is not pathogenic in intact rats, but after splenectomy it causes a severe anaemia which is usually fatal. The injections were repeated every three weeks during the experiment. The intact rats were given identical treatment to allow for any possible effects of neoarsphenamine on the variables under investigation.

Phenylhydrazine anaemia. Anaemia was produced by injecting a freshly prepared 2 p.c. solution of phenylhydrazine hydrochloride. Each rat was injected with 0·25 to 0·5 ml. solution at intervals of 3 to 7 days. The dosage was designed to maintain the haemoglobin value of the treated rats at approximately 60 p.c. of the value of the control rats. The haemoglobin value was determined on tail blood from 3 to 5 members of each group selected at random, and the mean of the values was regarded as representative of the whole group. Tests were made daily during the initial stages of treatment and weekly after the dosage had been established. Three weeks after the last phenylhydrazine injection, all the rats were tested and it was found that the mean haemoglobin values of the phenylhydrazine-treated rats were the same as those of the control rats.

Supplementary iron. The diet of all groups indicated in the scheme was supplemented with iron by giving a 2 p.c. solution of ferrous ammonium sulphate in 0·1 N hydrochloric acid as drinking water.

Collection of samples and analytical methods. The rats were anaesthetized with ether. The chest was opened and blood withdrawn from the heart with syringe and needle. The haemoglobin value was determined photometrically by the method of Walsh, Arnold, Lancaster, Coote and Cotter (1953). The liver and the spleen were dissected and weighed. The blood samples were centrifuged and the sera of rats of the same group pooled, each rat contributing an equal amount. Latent iron binding capacity was measured on aliquots of the fresh pooled sera. The remaining portion of the serum pools, the livers and the spleens were frozen and used at a later date for determining serum iron and non-haemoglobin tissue iron. The analytical methods have been described in other papers (Serum iron and iron binding capacity: Kaldor, 1953. Non-haemoglobin tissue iron: Kaldor, 1954b.).

RESULTS.

The results are summarized in three Tables. Table 1 shows the weight of the rats at the end of the experiment and the weight of their livers and spleens.

TABLE 1.

Mean Body Weights and the Mean Weights of Livers and Spleens.

1. Intact Rats					2. Splenectomized Rats			
Group	No.	Body weight gm.	Liver weight gm.	Spleen weight gm.	Group	No.	Body weight gm.	Liver weight gm.
1A	10	222	5·12	0·42	2A	10	222	5·11
1B	9	222	5·68	0·80	2B	10	217	5·98
1C	6	235	6·82	0·91	2C	10	223	6·42

Four rats in group 1C, and one rat in each of groups 1B, 2A, 2B and 2C, died before the end of the experiment. The deaths could not be ascribed to a single cause, but at autopsy several showed evidence of pneumonia. This condition is common in laboratory rats after the age of 6 months and is believed to be the result of infection secondary to the development of atelectatic foci in the lungs (Moise and Smith, 1929).

It will be seen from Table 1 that the weight of the liver was increased in both intact and splenectomized rats after treatment with phenylhydrazine (groups 1B and 2B) and was further increased in both intact and splenectomized rats receiving supplemental iron (groups 1C and 2C). The spleen weight of intact rats treated with phenylhydrazine (group 1B) was twice that of untreated rats (Group 1A). It was still greater in intact rats receiving supplemental iron during treatment with phenylhydrazine (group 1C), but the additional increase was small.

Haemoglobin values at the time the rats were sacrificed are shown in Table 2. Untreated splenectomized rats showed lower values than untreated intact rats. As a result of phenylhydrazine treatment the values of intact rats remained

TABLE 2.

Mean Haemoglobin, Serum Iron and Iron Binding Capacity Values and Saturation Indices.

1. Intact Rats						2. Splenectomized Rats					
Group	No.	Haemoglobin gm. per 100 ml. blood	Serum Iron	Total Iron Bndg. Capacity	Saturation Index (SI/TIBC) ×100	Group	No.	Haemoglobin gm. per 100 ml. blood	Serum Iron	Total Iron Bndg. Capacity	Saturation Index (SI/TIBC) ×100
			μg Fe per 100 ml. serum						μg Fe per 100 ml. serum		
1A	10	15·90	324	404	80	2A	10	15·39	278	428	65
1B	9	16·12	382	382	100	2B	10	16·24	254	454	56
1C	6	16·03	470	470	100	2C	10	16·87	389	389	100

TABLE 3.

Mean Iron Content of Livers and Spleens.

1. Intact Rats							2. Splenectomized Rats			
		Liver Iron		Spleen Iron		Liver + Spleen iron mg.			Liver Iron	
Group	No.	Concentration mg. per gm.	Total mg.	Concentration mg. per gm.	Total mg.		Group	No.	Concentration mg. per gm.	Total mg.
1A	10	0·69	3·55	2·93	1·23	4·78	2A	10	0·89	4·56
1B	9	1·26	7·18	4·89	3·91	11·09	2B	10	2·12	12·63
1C	6	2·58	17·55	7·63	6·92	24·47	2C	10	3·86	24·80

almost unchanged (groups 1B and 1C) whilst those of splenectomized rats increased (group 2B). A further increase in the latter rats occurred as a result of administration of supplemental iron (group 2C). The differences between the haemoglobin values of the three groups of splenectomized rats (groups 2A, 2B and 2C) are statistically significant. (Variance ratio, $F=7{\cdot}14$ with 2 and 27 degrees of freedom, $P=0{\cdot}01$–$0{\cdot}001$.)

On the other hand, the serum iron values were increased in intact as well as in splenectomized rats as a result of phenylhydrazine. The mean values of the intact rats were considerably higher than those of the splenectomized rats, but the significance of the differences could not be tested because the serum iron values of individual rats were not available.

The mean total iron binding capacity of the sera of the different groups remained within the limits of normal variation (Kaldor, 1954a). The iron binding capacity of the serum was saturated in all intact rats which were treated with phenylhydrazine, irrespective of the iron content of their diet. Saturation was not complete in splenectomized rats receiving only phenylhydrazine treatment (group 2B), but was complete in those splenectomized rats which received a combination of phenylhydrazine treatment and supplemental iron (group 2C).

The iron content of the organs is shown in Table 3. Phenylhydrazine treatment produced a large increase in the iron content of the livers and spleens of intact rats and of the livers of splenectomized rats (groups 1B and 2B). The addition of iron to the diet caused a further increase in these values (groups 1C and 2C). In the absence of the spleen, the liver seems to assume the storage function of this organ, because the difference between the iron content of the livers of identically treated intact rats and splenectomized rats was approximately equal to the amount of iron in the spleens of intact rats. This can be seen by comparing group 1A with group 2A, group 1B with group 2B, and group 1C with group 2C. The relationship may be expressed as

$$\text{Liver iron in splenectomized rats} = \text{Liver iron} + \text{spleen iron in intact rats.}$$

DISCUSSION.

This work was undertaken primarily to investigate the rôle of the spleen in the increased absorption of iron from the intestines which occurs during the anaemia phase produced by phenylhydrazine. Groups of intact and splenectomized rats were subjected to prolonged treatment with phenylhydrazine and the iron content of the livers of splenectomized rats was compared with the iron content of the livers and spleens of intact rats. The liver and spleen are the two chief organs for the storage of iron in the body, and both are able to store large amounts in excess of that found under physiological conditions.

It was shown that in splenectomized animals, the liver stores the iron which would normally be found in the liver and spleen of intact animals. This occurred in untreated animals as well as in those animals in which a large excess of iron was absorbed during phenylhydrazine-induced anaemia. It seems, therefore, that the spleen had little part in promoting excessive iron absorption under the conditions of the experiment. If this organ were important in this regard, the iron content of the liver of splenectomized animals would be less than the combined iron content of the liver and spleen of comparable intact animals.

The serum iron values are of interest. Although the splenectomized animals contained as much body iron as did the intact animals, the mean serum iron values of the splenectomized groups were considerably lower than those of the corresponding intact groups. In addition, the iron binding protein was only 56 p.c. saturated in the group of splenectomized rats which had been rendered chronically anaemic and had absorbed large amounts of iron. The protein was completely saturated in the corresponding group of intact rats. These findings are difficult to interpret, but will be further investigated in view of their importance in relation to the transport of iron in the blood.

SUMMARY.

It was reported that increased absorption of iron from the intestines occurred in rats during the anaemia produced by repeated injections of phenylhydrazine. In the present work the possibility was investigated that the spleen may have a rôle in promoting increased iron absorption during phenylhydrazine-induced anaemia. Such a possibility was considered as a result of certain observations made in the work referred to above.

It was shown that in splenectomized rats the liver stores the same amount of iron as the amount stored in the liver and spleen combined in intact rats. It was, therefore, concluded that the spleen has no active part in the increased iron absorption observed under the conditions of the experiment.

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REFERENCES.

Aufderheide, A. C., Horns, H. L. and Goldish, K. J. (1953): Blood, 8, p. 824.
Kaldor, I. (1953): Austral. J. exp. Biol., 31, p. 41.
Kaldor, I. (1954a): *Ibid.*, 32, p. 437.
Kaldor, I. (1954b): *Ibid.*, 32, p. 795.
Kaldor, I. (1954c): *Ibid.*, 32, p. 801.
Moise, T. S. and Smith, A. H. (1929): Proc. Soc. exp. Biol. Med., 26, p. 723.
Walsh, R. J., Arnold, B. J., Lancaster, H. O., Coote, M. A. and Cotter, H. (1953): A Study of Haemoglobin Values in New South Wales. N.H. and M.R.C. Special Report Series No. 5, Canberra.

STUDIES ON INTERMEDIARY IRON METABOLISM

VIII. THE FATE IN MICE OF INJECTED SACCHARATED OXIDE OF IRON

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(From the New South Wales Red Cross Blood Tranfusion Service).

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The present work arose from an earlier unpublished experiment in which attempts were made to increase within a short period the iron content of the liver and spleen of mice to several times the normal value. Saccharated oxide of iron, a colloidal preparation used for intravenous iron therapy, was chosen for injection. It was decided for technical reasons that the injections should be given intraperitoneally instead of by the more usual intravenous route. In the course of the work it became apparent that it is not possible to produce more than a slight increase of iron in the liver and spleen by this method.

This paper describes attempts to investigate the failure of intraperitoneally injected saccharated oxide of iron to produce a large increase in the visceral iron content. For purposes of comparison, other mice were injected intravenously with saccharated oxide of iron. As a result of this work some light has been cast on the fate of parenterally administered colloidal iron, and on the rate at which such iron can be converted to the physiological iron compounds of the body.

Materials and Methods.

Mice. Female mice were obtained for the experiment from the Section of Animal Genetics, C.S.I.R.O., University of Sydney. At the time of injection the average age and body weight of the mice was 60 days and 20 gm. respectively.

Saccharated oxide of iron. The commercial preparation "Ferrivenin-Benger," which contains 20 mg. iron per ml. solution, was diluted with distilled water as required. The iron content of Ferrivenin was checked by direct analysis.

Dosage. Ferrivenin was diluted one in ten with distilled water and 0·4 ml. (0·8 mg. iron) was injected into each mouse. This corresponds to an approximate dosage of 40 mg. iron per kg. body weight. Amounts of 0·1, 0·1 and 0·2 ml. were injected on three separate days with an interval of one day between injections.

Experimental procedure. The mice were divided into three groups.

Group 1. Sixteen mice received the Ferrivenin into the lateral tail vein.

Group 2. Twenty mice received the Ferrivenin into the peritoneal cavity.

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[1]This work was undertaken during tenure of a Fellowship from the National Health and Medical Research Council.

Three to five mice from each of these two groups were killed 1, 2, 4 and 10 weeks after the last injection.

Group 3. Sixteen control mice. As the mice were not fully grown at the time of injection, the control group was divided into two subgroups. Eight control mice were killed one week before the mice in groups 1 and 2 were injected; the remaining eight were killed eleven weeks after the last injection.

To obtain the material required for analysis the mice were anaesthetized with ether. The chest and abdomen were opened and blood for estimating the haemoglobin value was obtained by heart puncture. Exsanguination was completed by cutting the inferior caval vein. The liver and the spleen were then removed, weighed and stored in a freezing chamber. In some instances, to be specified below, the omentum and an aliquot of the parametrium were also removed and stored for analysis.

The immediate fate of intraperitoneally injected Ferrivenin. To trace the events following intraperitoneal injection of Ferrivenin, 0·2 ml. of the undiluted solution was injected into each of four mice. The mice were killed in groups of two, 90 and 180 minutes after injection. The abdominal cavity was opened, the volume of the peritoneal exudate was measured, and an unstained preparation of the exudate was examined under the microscope.

Analytical methods. The haemoglobin concentration of the heart blood was determined by photoelectric colorimetry as described by Walsh, Arnold, Lancaster, Coote and Cotter (1953). The iron content of the tissues was determined by a method described in another paper (Kaldor, 1954). This method measures non-haemoglobin iron only, and the values obtained in the measurements therefore represent the storage iron content of the tissues.

RESULTS.

The haemoglobin values will not be reported in detail because the haemoglobin values of control mice and those of injected mice did not differ significantly. It should be mentioned, however, that the haemoglobin values declined in mice of all three groups during the experiment. The difference between initial values and values obtained at the conclusion of the experiment was of the order of 2 gm. haemoglobin per 100 ml. blood. The reasons for this are not understood, but its bearing on the evaluation of the results of iron analyses will be discussed.

The results of iron analyses of livers and spleens are shown in Table 1 and Fig. 1. The following features should be noted:

Control group. The initial iron content of the liver was doubled and there was also a small rise in spleen iron during the experiment. These changes are the result of body growth accompanied by the normal accumulation of iron.

Intravenously injected group. One week after intravenous injection of Ferrivenin, the iron content of both liver and spleen was 9 to 10 times higher than in the control mice. The iron content gradually decreased after the end of the first week, but at the tenth week the iron content was still four times higher in the liver and seven times higher in the spleen than in the control mice.

Intraperitoneally injected group. One week after the intraperitoneal injection of Ferrivenin, the iron content of the liver was only twice as high as in the control mice, and that of the spleen about 50 p.c. higher. In contrast to

TABLE 1.

Non-haemoglobin Iron in the Liver and Spleen of Mice injected with Saccharated Oxide of Iron.

Time elapsed since injections	Route of administration	Number of mice	Mean body weight: grams	Iron content of liver: micrograms		Iron content of spleen: micrograms	
				Mean	Range	Mean	Range
Control mice, killed one week before injections		8	19	68	46-99	9	5-13
1 Week	Intravenous	5	22	958	830-1100	89	67-112
	Intraperitoneal	5	21	174	149-191	14	12-16
2 Weeks	Intravenous	3	24	742	662-823	77	65-84
	Intraperitoneal	5	25	186	131-262	16	7-21
4 Weeks	Intravenous	5	25	879	833-942	75	58-90
	Intraperitoneal	5	25	212	149-300	20	15-30
10 Weeks	Intravenous	3	26	611	570-646	69	52-102
	Intraperitoneal	5	27	307	163-490	43	32-58
Control mice killed eleven weeks after injections		8	28	149	105-214	12	11-14

the intravenously injected animals, the iron content of the liver gradually and slowly rose during the remainder of the experiment, but the net gain of iron in the liver was small. In the spleen there was a similar but greater gain, but it must be remembered that there is roughly only one-tenth as much iron in the spleen as in the liver.

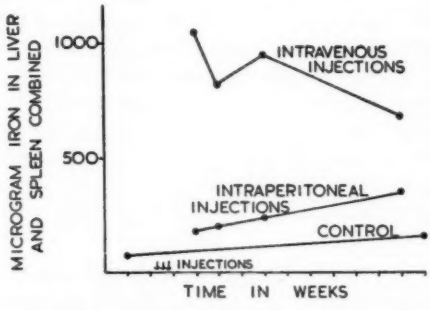

Fig. 1. Non-haemoglobin iron in the liver and spleen of mice injected with saccharated oxide of iron.

The fate of Ferrivenin injected intraperitoneally. Owing to the dark brown colour of Ferrivenin, its fate after injection can be traced without difficulty. Ninety minutes after the injection of 0·2 ml. undiluted Ferrivenin into the peritoneum, 0·5 to 0·7 ml. turbid brown exudate was recovered from the peritoneal cavity. The exudate contained a rapidly sedimenting phase. Examined under the microscope the sediment consisted of coarse aggregates of brown precipitate and a variety of cellular elements, including large, irregularly shaped phagocytic cells, heavily loaded with precipitate. The volume of peritoneal exudate and its composition was similar in mice killed 180 minutes after injection of 0·2 ml. Ferrivenin into the peritoneum.

Macroscopic deposits of precipitated Ferrivenin were seen on the peritoneal membranes of all mice killed 1 to 10 weeks after the intraperitoneal injections. Deposits were heaviest on the omental membranes, the mesentery and the parametrium. The parietal peritoneum was diffusely stained. In contrast, the peritoneal membranes of mice injected intravenously showed no macroscopic deposits at any stage after injection.

An attempt was made to compare the iron content of the peritoneal membranes of intraperitoneally injected mice with that of intravenously injected animals. Aliquots of the membranes were taken ten weeks after injection from identical locations in the two groups of mice, and the iron content determined. The iron content of peritoneal aliquots of intravenously injected mice was 6 to 9 μg. per aliquot, whereas the corresponding figure for the intraperitoneally injected mice was 20 to 100 μg. per aliquot.

DISCUSSION.

It has been shown repeatedly (Capell, 1930; Nissim, 1949) that intravenously injected saccharated oxide of iron is rapidly removed from the circulation by phagocytes and that most of the injected material is taken up by the reticuloendothelial cells of the liver. According to histochemical evidence, the iron persists in the phagocytic cells for a long time (Nissim, 1953).

In the present experiments, the iron content of the livers was nearly ten times as much one week after intravenous injection with Ferrivenin as in control mice. Subsequent assays showed a gradual fall. Ten weeks after injection, the mice had only four-and-a-half times as much iron in their livers as the control mice. In another unpublished experiment, mice received intravenous injections with twice as much Ferrivenin as in the present experiment. A 24-fold increase occurred in liver iron in one week, and ten weeks after the injections the iron content of the livers was only thirteen times as much as in control mice. It appears from this that the amount of Ferrivenin initially deposited in the liver after intravenous injection determines the rate at which iron subsequently disappears from this organ. A similar conclusion was reached by Stevens, White, Hegsted and Finch (1953), who investigated iron excretion in mice with the aid of radio-iron.

It seems unlikely that an appreciable portion of the iron in the liver could have been removed and deposited elsewhere in the body. It can also be assumed that the iron which disappeared from the liver was not required for haemoglobin synthesis, because the circulating haemoglobin mass was practically unchanged in the mice from the beginning of the experiment to the end. Any increase in the haemoglobin mass due to growth was offset by the gradual decline of the haemoglobin concentration which occurred in both injected and control mice. It must be concluded that the iron deposited in the liver after intravenous injection of Ferrivenin was gradually eliminated from the body.

Intraperitoneally injected Ferrivenin was also rapidly phagocytosed within

the abdominal cavity and the phagocytosed material became the source of a slow but steady rise in visceral iron. The iron content of the livers was doubled and that of the spleens was increased fourfold in ten weeks after intraperitoneal injection. At this stage the iron content was still rising in both organs and assays of the peritoneal membranes performed at ten weeks showed that a considerable portion of the Ferrivenin initially deposited there still persisted and remained a possible source of further increases in visceral iron.

It may be assumed that if the phagocytes release any intact particles of Ferrivenin within the abdominal cavity, these particles would be reingested by other phagocytes before they could reach the liver or the spleen. The saccharate molecule must first be broken up, the iron released and then passed on to the iron binding protein of the blood for transport. In this protein bound form, the Ferrivenin iron loses its identity and becomes available for metabolism by the parenchymal cells of the liver in the same way as iron released from the deranged haemoglobin molecules, or iron absorbed from the intestines.

SUMMARY.

Saccharated oxide of iron was injected intravenously into a group of mice. Another group of mice was injected intraperitoneally with the same preparation in the same dosage.

A large increase occurred in the non-haemoglobin iron content of the liver and spleen one week after intravenous injection. This initial increase was followed by a gradual decrease in the iron content of these organs. Only about one-half of the iron initially deposited in the liver remained there ten weeks after injection.

Intraperitoneally injected saccharated oxide of iron was precipitated and phagocytosed within the peritoneal cavity. The precipitated material persisted in the peritoneal membranes throughout the period of observation and became the source of a slow, gradual rise in liver and spleen iron.

Acknowledgment. The author wishes to acknowledge the assistance of Miss Marjorie Powell with all technical aspects of this work.

REFERENCES.

Cappell, D. F. (1930): J. Path. Bact., 33, p. 175.

Kaldor, I. (1954): Austral. J. exp. Biol., 32, p. 795.

Nissim, J. A. (1949): M.D. Thesis, University of London. Quoted by Nissim, J. A. (1953): See below.

Nissim, J. A. (1953): J. Path. Bact., 66, p. 185.

Stevens, A. R., Jr., White, P. L., Hegsted, D. M. and Finch, C. A. (1953): J. biol. Chem., 203, p. 161.

Walsh, R. J., Arnold, Barbara J., Lancaster, H. O., Coote, Margaret A. and Cotter, Helen (1953): A Study of Haemoglobin Values in New South Wales, with observations on haematocrit and sedimentation rate values. N.H. and M.R.C. Special Report Series No. 5, Canberra.

CARDIAC ACTIONS OF COENZYME I.

I. ACTIONS ON THE GUINEA-PIG AND RAT HEART *IN SITU* AND ON THE ISOLATED GUINEA-PIG HEART

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The vasodepressor activity of crude coenzyme I preparations was first described in 1931 by Euler and Gaddum. Later, Euler, Euler and Schlenk (1940) compared the activity of a pure sample of coenzyme I with adenosine on the blood pressure in the cat and on rabbit and guinea-pig smooth muscle, showing that it possessed the same pharmacological properties as other adenyl compounds. However, an important feature of the action of the adenyl compounds is their effect on cardiac rhythm. The similarity between the other properties of coenzyme I and the adenyl compounds warrants an extension of these comparisons to include this aspect.

The cardiac actions of coenzyme I in the guinea-pig and rat have therefore been compared with those of adenosine. In an earlier paper (Rand, Stafford and Thorp, 1955), mention was made of the heart block produced in the guinea-pig by coenzyme I. Preliminary experiments indicated that there was a great difference in the slopes of the adenosine and coenzyme I dose-response curves. The quantitative aspects of the dose-response relationships for these drugs have therefore been the subject of a more detailed study in the work to be described.

METHODS.

Intra-atrial injection in the guinea-pig and rat. Guinea-pigs were anaesthetized with 25 p.c. urethane (0·7 ml. per kg.) and rats with nembutal (50 mg./kg.). Equimolar solutions of the adenyl compounds in 0·9 p.c. NaCl were placed in 20 ml. syringes each operated by a motor-driven screw. Injections were given at 2-minute intervals through 1 mm. polythene tubing which was tied into the left atrium. The dose to be injected was regulated by setting a thyratron timing relay which activated the injection apparatus for any predetermined interval. The response, measured from E.C.G. records, was counted as the time in seconds

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during which heart block was present. Solutions of coenzyme I (Sigma 90) in 0·9 p.c. NaCl were adjusted to pH 7·0 with 0·02 N NaOH and diluted to contain the equivalent of 1 mg. of adenosine per ml. Coenzyme solutions were stored at 0° C. and fresh solutions were prepared weekly.

Isolated guinea-pig heart. The Langendorff preparation of the guinea-pig heart was perfused with Ringer-Locke solution at 29° C. The drugs were dissolved in Ringer-Locke and injected into the cannula as close as possible to the aorta.

Throughout the experimental section, doses of coenzyme I are expressed in terms of their adenosine content (2·48 mg. of coenzyme I=1 mg. of adenosine).

RESULTS.

Action of coenzyme I on the guinea-pig heart in situ.

On intra-atrial injection, coenzyme I (*ca.* 50 μg.) produced a transient period of partial A–V block accompanied by sinus bradycardia. The configuration of the E.C.G. was in no other way altered. When the actions of coenzyme I and adenosine were compared by giving alternate intra-atrial injections, the following differences were observed:

(i) The latent period between the moment of injection and the beginning of the response was longer with coenzyme I than with an equi-active dose of adenosine.

(ii) Coenzyme I, when compared with a dose of adenosine which produced a response of the same total duration, gave rise to a lower grade heart block.

(iii) Unlike adenosine, coenzyme I caused a gradual increase in sensitivity as a result of injections given at regular intervals.

(iv) Coenzyme I was less active than adenosine in low doses and more active in high doses.

To obtain a quantitative comparison between adenosine and coenzyme I, dose-response curves were determined using 4 dose levels for each substance. For these experiments, 2 separate cannulae from 2 motor-driven syringes were tied into the left atrium. The injections were arranged in random order. The results of 5 experiments are summarized in Table 1. In each comparison, the

TABLE 1.

Comparison of the regression coefficients for the dose-response curves of adenosine and coenzyme I.

Exp. no.	Adenosine Slope ± S.E.	Coenzyme I Slope ± S.E.	"t"	D.F.	Significance
1	24·8 ± 2·24	56·1 ± 3·96	7·04	40	$P<0·001$
2	33·9 ± 2·82	122·3 ± 12·89	8·00	32	$P<0·001$
3	45·6 ± 4·77	115·5 ± 7·17	8·13	40	$P<0·001$
4	10·9 ± 4·14	95·8 ± 29·89	3·61	40	$P=0·001$
5	31·6 ± 4·73	80·1 ± 20·76	2·80	48	$0·001<P<0·01$

slope of the log. dose-response curve was much higher for coenzyme I than for adenosine, and these differences were shown to be highly significant when analyzed by the "t"-test. A typical experiment is illustrated in Fig. 1, from which it can be seen that the log. dose-response relationship which was used in calculation of the regression coefficient provided a satisfactory fit to the data. The regressions were tested by an analysis of variance and found to be highly significant. It is clear, therefore, that no valid estimate can be made of the relative potency of coenzyme I compared with adenosine. In each of the comparisons, the point of intersection of the dose-response lines for adenosine and coenzyme I was determined. The corresponding doses fell close together between 33 and 45 μg. (expressed in terms of adenosine) and the responses ranged from 10 to 21 seconds of heart block. It is only in this region that equimolar doses are equi-active; with higher doses, coenzyme I is more active and with lower doses adenosine is more active.

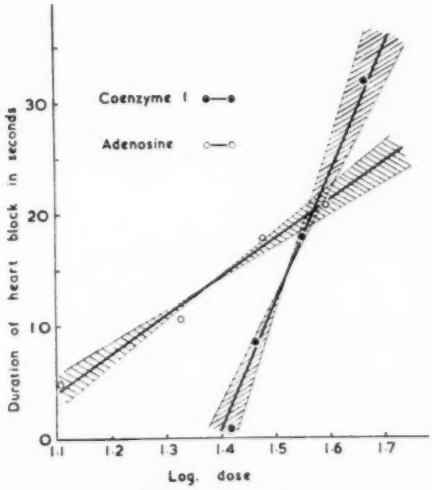

Fig. 1. Log. dose-response curves for adenosine and coenzyme I determined on the same animal. The shaded areas represent the fiducial limits of the regression coefficients at P=0·05.

Action of coenzyme I on the isolated guinea-pig heart.

Qualitatively, both adenosine and coenzyme I produced slowing of the heart rate and heart block, but, in every case, adenosine produced a more clear-cut effect. The responses to 3 dose levels of adenosine and coenzyme I are shown in Fig. 2. When the response was measured as the time (in seconds) for the heart

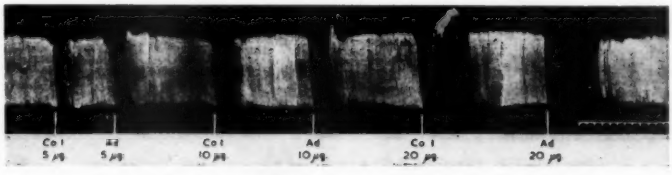

Fig. 2. Isolated guinea-pig heart. Doses of coenzyme I are expressed in terms of their adenosine content. The time marker indicates 10-second intervals.

beat, amplitude and rate to return to normal, comparison of adenosine and coenzyme I showed that equimolar doses were equi-active over a wide range. In 4 experiments, an attempt was made to determine the regression coefficients of these adenyl compounds on the Langendorff heart. In 3 of these experiments, the slopes of the regression lines were determined but could not be shown to be significant by analysis of variance. In the remaining experiment, the regression coefficients for adenosine and coenzyme I were 142±31·62 and 113±35·64 respectively. These slopes were not significantly different when analyzed by the "t"-test (t=0·482, 26 degrees of freedom). The relative potency of coenzyme I was 94 p.c. of adenosine in this comparison.

Action of coenzyme I on the rat heart in situ.

In the rat, as in the guinea-pig, regular injections of adenosine produce well defined periods of heart block which are reproducible and easily measured. Coenzyme I produced a qualitatively similar response to adenosine in the rat. However, with responses to adenosine and coenzyme I of the same total duration, adenosine produced a higher grade of heart block and had a somewhat shorter latent period. Dose-response curves for adenosine and coenzyme I were determined in the rat using methods described above for the guinea-pig. The results are summarized in Table 2. In no case was there any significant difference in

TABLE 2.

Comparison of Coenzyme I and Adenosine in the Rat.

Exp. No.	Regression coefficient		"t" (degrees of freedom)	Significance of difference	Relative potency of Co I p.c.
	Adenosine	Coenzyme I			
21	19·7	29·0	1·599 (32)	$0·1<P<0·2$	34·4
22	28·0	15·8	1·325 (40)	$0·1<P<0·2$	77·0
25	24·3	27·9	0·436 (25)	$0·6<P<0·7$	53·5
26	33·1	34·6	0·600 (60)	$0·5<P<0·6$	79·3

the slopes of the log. dose-response curves of the two substances when the regression coefficients were compared by the "t"-test. The potency of coenzyme I relative to adenosine has been calculated from the data, and is shown in the last column of Table 2. The potency ratio in the Table is determined on a molar basis; on a weight basis the relative potency varied from 14 to 32 p.c.

DISCUSSION.

The action of coenzyme I on the guinea-pig heart is, in general, the same as that of adenosine, adenylic acid and A.T.P. (see review by Drury, 1936); small doses produce sinus bradycardia and lengthening of the P–R interval, while with larger doses there is a transient period of partial heart block. In

the case of adenosine, adenylic acid and A.T.P., these actions are identical (Rand, Stafford and Thorp, 1955); however, the response to coenzyme I differs qualitatively in two ways: the latent period is longer, and the heart block is not as clear-cut. These slight but definite differences between the action of coenzyme I and adenosine on the guinea-pig heart *in situ* are also seen in the isolated guinea-pig heart and on the rat heart *in situ*.

Coenzyme I has been compared with adenosine or adenylic acid on the depressor action on the rabbit blood pressure (Euler, Euler and Schlenk, 1940; Euler and Gaddum, 1931; Gard, 1931), on the isolated rabbit intestine (Euler *et al.*, 1940; Euler and Gaddum, 1931), on the guinea-pig isolated uterus (Euler *et al.*, 1940; Euler and Gaddum, 1931) and on the isolated rabbit heart (Gard, 1931). These workers have stated that adenosine and coenzyme I have the same qualitative effects, although Euler *et al.* (1940) indicated that coenzyme I was somewhat inconstant in its action on the rabbit blood pressure and that the rate of development of the depressor response was slower.

Quantitative comparisons between coenzyme I and the other adenyl compounds do not seem to have been carried out by the accepted biological assay techniques, but a number of semi-quantitative observations have been made. Cozymase was found to have 60 p.c. of the potency of adenosine by weight when tested on the rabbit blood pressure and the guinea-pig uterus (Euler and Gaddum, 1931) and was not quite as active as yeast adenylic acid (Gard, 1931). With a more purified preparation, Euler *et al.* (1940) stated that cozymase was slightly less active than adenosine by weight in its depressor action on the rabbit blood pressure and on the isolated rabbit intestine, but they were equi-active on the guinea-pig uterus. Our work has indicated that on the guinea-pig heart *in situ*, a quantitative comparison between adenosine and coenzyme I is not possible because of the marked difference in slope of the dose-response curves. On the isolated guinea-pig heart, there was no difference in potency, and on the rat heart *in situ*, the relative potency of coenzyme I expressed on a molar basis ranged from 30–80 p.c. of adenosine.

The question arises whether coenzyme I exerts its pharmacological action as such, or whether it is broken down first into a simpler phosphorylated adenosine derivative and acts in this form. The latter hypothesis is supported by Gard (1931), who found that coenzyme I and its enzymatically split products were equi-active on the rabbit blood pressure and the isolated heart, and also by Euler *et al.* (1940). However, if coenzyme I has 60 p.c. of the activity of adenosine (Euler and Gaddum, 1931) or the same activity as adenosine (Euler *et al.*, 1940) when compared on a weight basis, it would be expected to have 150 to 250 p.c. of the activity of adenosine on a molar basis. It is difficult to see how the adenosine derived from the breakdown of coenzyme I would be more active than adenosine itself. The obvious conclusion is that coenzyme I is acting as such, and that it has the same general properties as adenosine, but to a higher

degree. The findings reported in this paper that the slopes of the dose-response curves of adenosine and coenzyme I are different and that high doses of coenzyme I are more active than equimolecular doses of adenosine, suggest that coenzyme I is acting as such. The slight qualitative differences between coenzyme I and adenosine also offer support for the hypothesis that coenzyme I itself has pharmacological actions.

SUMMARY.

Coenzyme I produces a transient period of bradycardia and A–V block when tested on the guinea-pig and rat heart *in situ* and on the isolated guinea-pig heart. On the guinea-pig heart *in situ*, the slope of the dose-response curve for coenzyme I was significantly steeper than for adenosine, so no quantitative comparison could be made. On the isolated guinea-pig heart, equimolar doses of coenzyme I and adenosine were equi-active. On the rat heart *in situ*, coenzyme I had about 50 p.c. of the activity of adenosine when expressed on a molar basis.

REFERENCES.

Drury, A. N. (1936): Physiol. Rev., 16, p. 292.
Euler, H. v., Euler, U. S. v. and Schlenk, F. (1940): Acta physiol. Scand., 1, p. 19.
Euler, U. S. v. and Gaddum, J. H. (1931): J. Physiol., 72, p. 74.
Gard, S. (1931): Z. physiol. Chem., 196, p. 65.
Rand, M., Stafford, A. and Thorp, R. H. (1955): Austral. J. exp. Biol.

CARDIAC ACTIONS OF COENZYME I

II. FACTORS AFFECTING THE RESPONSE OF THE GUINEA-PIG HEART TO COENZYME I

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(Accepted for publication 17th August, 1955.)

In the preceding paper (Stafford and Rand, 1955), it has been shown that the pharmacological actions of coenzyme I on the guinea-pig heart bear a marked resemblance to those of adenosine. The modification of the cardiac response to coenzyme I by vagotomy, prostigmine, magnesium and quinidine has therefore been investigated since these agents are known to affect the response to other adenyl compounds (Green and Stoner, 1950). The effect of ouabain on the heart block produced in the guinea-pig by adenosine has been described previously (Rand, Stafford and Thorp, 1955a)—a dose of ouabain too small to cause electrocardiographic changes produces a marked and long-lasting potentiation of the response to adenosine. When this potentiation by ouabain was extended to other adenyl compounds, it was found that adenylic acid and A.T.P. were potentiated to the same extent as adenosine, but coenzyme I was potentiated to a much greater extent (Rand, Stafford and Thorp, 1955b). In order to explain this difference, an investigation of the slopes of the dose-response curves for adenosine and coenzyme I before and after the administration of ouabain has been undertaken.

METHODS.

Regular intra-atrial injections of coenzyme I were given at 2-minute intervals for the duration of each experiment. The response was recorded on the E.C.G. The details of the methods have already been described (Stafford and Rand, 1955). Quinidine sulphate (Burroughs Wellcome), ouabain (Burroughs Wellcome) and prostigmine methyl sulphate (Roche) were dissolved in 0·9 p.c. NaCl. Magnesium sulphate was made up in an isotonic solution (0·154 M). These drugs were injected into the jugular vein.

The results are taken from 16 guinea-pigs weighing between 300 and 400 gm.

RESULTS.

The rôle of the vagus in the response to coenzyme I. In 5 experiments, the cardiac response to coenzyme I was not appreciably altered by right, left or bilateral vagotomy. Following the injection of 10 μg./kg. of prostigmine in a

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guinea-pig with intact vagi, the period of heart block produced by coenzyme I was increased by 50–75 p.c.; at the same time, the P–R interval was increased from 0·045 to 0·055 sec. When the vagi were cut, the P–R interval dropped to 0·040 sec., and the response to coenzyme I returned to its original level.

Quinidine. Intravenous injection of 3 to 15 mg./kg. of quinidine sulphate resulted in a decreased duration of the heart block produced by injection of coenzyme I. Fig. 1 shows that the inhibiting action of quinidine on the response

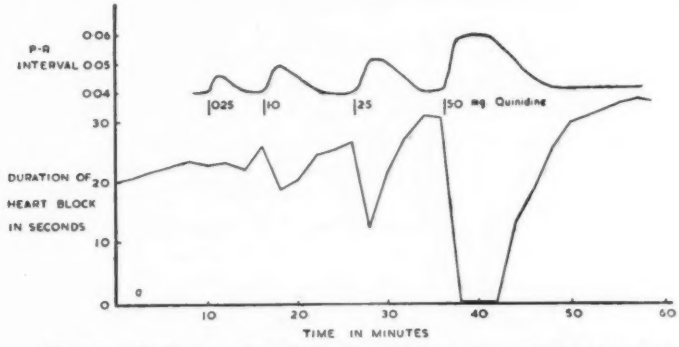

Fig. 1. The lower curve shows the responses to successive injections of the same dose of coenzyme I given at 2-minute intervals. The upper curve shows the P–R interval in seconds. Injections of quinidine were given as indicated by the strokes between the two curves.

to coenzyme I is proportional to the dose of quinidine injected. In the upper part of Fig. 1, the increase in the duration of the P–R interval produced by the injections of quinidine is shown. The prolongation of the P–R interval by quinidine is directly proportional to the degree of antagonism of coenzyme I. As the effect of quinidine wore off, the return of the response to coenzyme I to its original level ran parallel to the return of the P–R interval.

Magnesium. Following the intravenous injection of 100 mg./kg. of magnesium sulphate there was a two-fold increase in the duration of the response to coenzyme I. At the same time there was an increase in the length of the P–R interval. The response to coenzyme I returned to its original level within three to five minutes, while the P–R interval remained long.

Ouabain. The effect of ouabain on the cardiac response to coenzyme I has been examined quantitatively. Injections of 3 dose levels were arranged in random order and given at 2-minute intervals. The responses were measured and from these, the slope of the dose-response line for coenzyme I was calculated. Then ouabain was given by intravenous infusion until the response to coenzyme I was markedly prolonged. The injection of ouabain was stopped and while the response remained at this high level, the slope of the dose-response line was again determined in the same way. Both before and after ouabain, 6–8 replicates of each dose level were made. For purposes of comparison a similar series of

experiments was carried out with adenosine and ouabain. Table 1 shows the regression coefficients for the log. dose-response lines of coenzyme I and adenosine before and after ouabain. In each case, the regression was shown to be highly significant by analysis of variance. Injection of ouabain made no significant difference to the slope of the dose-response line (Table 1) although there was an over-all sensitization of the animal to the adenyl compound (Fig. 2).

TABLE 1.

Exp. No.	Adenyl compound	Regression		"t"	P
		Initial	After ouabain		
6	Adenosine	22·7	26·6	1·63	0·1-0·2
7	Adenosine	20·8	26·5	1·32	0·1-0·2
9	Coenzyme I	81·8	78·8	0·14	0·8-0·9
11	Coenzyme I	108·8	99·6	0·31	0·7-0·8

The four experiments shown in Table 1 have been analyzed to determine the degree of potentiation and also the ratio of doses before and after ouabain. The dose ratio is the ratio of doses producing the same duration of heart block after and before ouabain, and is independent of the actual dose selected for comparison. It is obtained from the antilog. of the distance M, shown in Fig. 2, expressed as a percentage. The degree of potentiation is the ratio of the responses produced by the same dose of adenyl compound after and before the injection of ouabain, and will therefore depend on the initial length of the response produced by the chosen dose of adenyl compound. In order to provide figures comparable with previously published results (Rand *et al.*, 1955b) the dose which initially produced a 4-second response was selected for the calculation. The degree of potentiation was determined from the regression lines shown in Fig. 2 by the measurement of the distance R, from the relation-

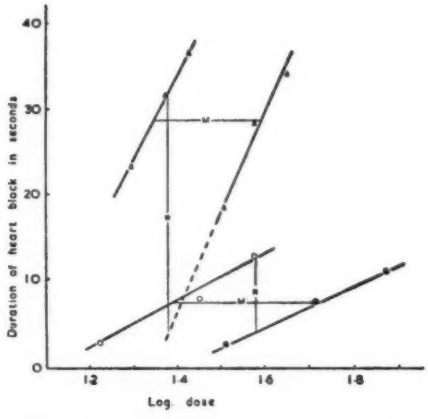

Fig. 2. Log. dose-response curves for adenosine and coenzyme I in the guinea-pig before and after the injection of ouabain.

●——● Adenosine before ouabain.
O——O Adenosine after ouabain.
▲——▲ Coenzyme I before ouabain.
△——△ Coenzyme I after ouabain.

The ratio of doses producing the same response before and after ouabain is indicated by the lines marked M. The degree of potentiation of a 4-second response by ouabain is indicated by the lines marked R. The doses of coenzyme I are shown in terms of their adenosine content.

ship: degree of potentiation $=\frac{R+4}{4}\times 100$ p.c. The ratio of the doses producing equal responses after and before ouabain is substantially the same for adenosine and coenzyme I. However, the degree of potentiation of the 4-second response is about threefold in the case of adenosine and eightfold for coenzyme I. These results are summarized in Table 2.

TABLE 2.

Exp. No.	Adenyl compound	Percent. potentiation of 4-sec. response	Ratio between doses required to produce same response after and before ouabain
6	Adenosine	305	0·517 : 1
7	Adenosine	260	0·448 : 1
9	Coenzyme I	750	0·390 : 1
11	Coenzyme I	775	0·612 : 1

DISCUSSION.

The influence of various factors on the response of the guinea-pig heart to coenzyme I is in agreement with published results for other adenyl compounds. In the guinea-pig, the vagus plays an insignificant part in the cardiac response to adenosine and A.T.P. (Wayne, Goodwin and Stoner, 1949); we have confirmed this finding for adenosine and extended it to coenzyme I. The slight but definite potentiation of coenzyme I by prostigmine agrees with Drury, Lutwak-Mann and Solandt (1938) who found that this drug slightly potentiates the action of adenosine. The antagonism of the response to A.T.P. on the guinea-pig heart by quinidine, reported by Green and Stoner (1950), has been extended to adenosine and coenzyme I. The transient nature of this antagonism is probably due to the rapid elimination of quinidine (Weiss and Hatcher, 1927). The potentiation of the action of coenzyme I by magnesium sulphate injection agrees with the finding of Wayne *et al.* (1949) with A.T.P. It is interesting to note the different ways in which these agents alter the response of the guinea-pig to coenzyme I and the other adenyl compounds in relation to their effects on the atrioventricular conduction time as judged from the P–R interval. Thus magnesium, prostigmine and quinidine all prolong the P–R interval, but only the first two agents potentiate the action of adenyl compounds. It would have been expected that prolongation of the P–R interval would sum with the effect of the adenyl compounds and produce a potentiation of the heart block. That this is not so in the case of quinidine suggests that quinidine and the adenyl compounds prolong the P–R interval by different mechanisms.

The effect of ouabain on the response to coenzyme I in the guinea-pig has been reported earlier by Rand *et al.* (1955b), who found that coenzyme I was potentiated to a much greater extent by ouabain than was adenosine. In order

to explain this effect it was suggested that some additional mechanism was implicated, such as inhibition of the breakdown of coenzyme I. However, it has been shown here that although the response to a given dose of coenzyme I is potentiated to a much greater extent by ouabain than is the response to adenosine, the ratio between the doses producing the same effect before and after the injection of ouabain is the same in the case of adenosine and coenzyme I. The explanation of the apparent difference between coenzyme I and adenosine is then simply that the dose-response curves have a different slope and that the sensitivity of the preparation as a whole and not the slope is altered by ouabain.

SUMMARY.

The heart block produced by injection of coenzyme I in the guinea-pig is not affected by vagotomy. Quinidine antagonizes the response to coenzyme I. Magnesium sulphate and prostigmine give a slight potentiation of the response and ouabain produces a marked and long-lasting potentiation.

Acknowledgments. We wish to thank Dr. E. J. de Beer, of the Wellcome Research Laboratories, Tuckahoe, N.Y., for the gift of coenzyme I used in these experiments. Grateful acknowledgment is made to Professor R. H. Thorp for his advice and encouragement during the course of these investigations.

REFERENCES.

Drury, A. N., Lutwak-Mann, C. and Solandt, O. M. (1938): Quart. J. exp. Physiol., 27, p. 215.
Green, H. N. and Stoner, H. B. (1950): "Biological Actions of the Adenine Nucleotides." Lewis, London.
Rand, M., Stafford, A. and Thorp, R. H. (1955a): J. Pharm. exp. Ther., 114, p. 119.
Rand, M., Stafford, A. and Thorp, R. H. (1955b): Austral. J. exp. Biol.
Stafford, A. and Rand, M. (1955): Austral. J. exp. Biol.
Wayne, E. J., Goodwin, J. F. and Stoner, H. B. (1949): Brit. Heart J., 11, p. 55.
Weiss, S. and Hatcher, R. A. (1927): J. Pharm. exp. Ther., 30, p. 335.

THE EFFECT OF CARDIAC GLYCOSIDES ON THE HEART BLOCK PRODUCED IN THE GUINEA-PIG BY ADENOSINE AND ITS DERIVATIVES

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(Accepted for publication 17th August, 1955.)

The adenine nucleotides are better known for their rôle in intracellular biochemical processes than for their pharmacological properties. This may explain why the effect of the cardiac glycosides on the utilization and production of A.T.P. in heart muscle has been the subject of many investigations (see reviews by Wollenberger, 1949; Loubatieres, 1951), but little work has been devoted to the inter-relationship between cardiac glycosides and the adenyl compounds on a pharmacological level. Fischer and Langemann (1951) have demonstrated that pre-treatment of the isolated frog heart with adenylic acid or A.T.P. reduced the amount of cardiac glycoside required to increase its systolic strength and to produce systolic standstill. Rand, Stafford and Thorp (1955) have shown that the duration of the heart block brought about by adenosine in the guinea-pig is increased about four-fold by a dose of ouabain which in itself is too small to produce any observable changes in the E.C.G. Larger doses of ouabain, though still not affecting the configuration of the E.C.G., changed the response to injections of adenosine from one of heart block to the precipitation of a transient period of electrocardiographic irregularities which were identical with the characteristic effects of cardiac glycoside intoxication. When doses of ouabain large enough to produce electrocardiographic changes were reached, the injection of adenosine was without any effect. These findings could not be dismissed as a mere summation of the actions of adenosine and ouabain on the P–R interval, and suggested that ouabain interfered specifically with the detoxification of adenosine. It seemed of interest to find whether this action was confined to adenosine, or whether an explanation of the mechanism of the potentiation could be found by an examination of other chemically related derivatives.

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The action of adenylic acid, A.T.P. and coenzyme I on the guinea-pig heart has therefore been compared with that of adenosine, and the potentiation of this action by various other cardiac glycosides is described.

METHODS.

Intra-atrial injections in the guinea-pig. Adenosine and related drugs were injected into the left atrium and the E.C.G. recorded as described previously (Rand *et al.*, 1955). The anaesthetic used was urethane (1·6 gm./kg.) and the animals were artificially respired. An automatic apparatus was used to control the interval between injections of the adenyl compounds and the rate and the volume of the injection. A mechanical time switch with an approximately 2-minute cycle of operation was used to fix the interval between injections. The volume of injected solution was determined by a thyratron timing relay controlling a motor-driven syringe for any pre-set time interval. With this device, volumes of 0·01–0·1 ml. of solution could be injected by pulses of 2–10 seconds duration. The time switch also started the paper drive of the E.C.G. permitting automatic recording of the response. Adenosine, 5-adenylic acid, A.T.P. (Light) and coenzyme I (Co I) (Sigma 90) were made up in neutralized equimolar solutions in 0·9 p.c. NaCl to contain the equivalent of 1 mg./ml. adenosine. Alcoholic solutions of the cardiac glycosides, ouabain, digitoxin, digoxin and lanatoside C were diluted for use with 0·9 p.c. NaCl and injected into the jugular vein. The maximum alcohol concentration injected was 1·0 p.c.

The results are taken from experiments on fifty guinea-pigs.

RESULTS.

The actions of adenosine, adenylic acid, A.T.P. and coenzyme I on the guinea-pig E.C.G.

The actions of adenylic acid and adenosine were compared by injecting equimolecular amounts of each drug alternately at two-minute intervals through two separate left atrial cannulae. Small doses of adenylic acid (10–20 μg.) produced exactly the same effect as an equimolecular dose of adenosine, *viz.*, bradycardia and a prolongation of the P–R interval. Similarly, the duration of the heart block produced by a larger dose of adenosine (20–50 μg.) was exactly equal to that produced by an equivalent dose of adenylic acid. A.T.P. was also compared with adenosine by the same method, and gave exactly the same qualitative and quantitative results as were found with adenylic acid. However, when coenzyme I was tested in this way, it was found to produce a qualitatively similar response to adenosine, but it was only at certain dose levels that equimolecular doses produced periods of heart block of the same duration. In the experiments described below, the dose of the adenyl compounds was adjusted to give a response lasting approximately four seconds. The duration of the response was taken to be the total period of time measured from R-wave to R-wave during which heart block was present.

The effect of ouabain.

When the response to 20 alternate injections of equi-active doses of adenosine and adenylic acid had been determined, a constant infusion of ouabain (1·5 μg./kg./min.) was commenced. The duration of the heart block produced by both adenosine and adenylic acid increased steadily until a maximum potentiation was reached about 40 minutes after starting the injection of ouabain. At this stage, there was no evidence of irregularities in the E.C.G. attributable to the ouabain. If the administration of ouabain was continued, the response to both adenosine and adenylic acid changed simultaneously from a heart block to the precipitation of a transient period of electrocardiographic irregularities identical with those which were produced by the ouabain alone at an even higher dose level. These irregularities produced by injection of the adenyl compounds lasted the same length of time as the previous response of heart block, and the recovery was complete. At a higher dose level of ouabain, when the control E.C.G. showed characteristic signs of cardiac glycoside toxicity, the injections of both adenosine and adenylic acid were entirely without effect. The course of the first part of these experiments is shown in the top panel of Fig. 1, where the duration of the heart block produced by regular injections of adenosine and adenylic acid is plotted against time, and therefore also against the dose of ouabain. Each point on the graphs represents the mean of observations on 3 separate guinea-pigs which were treated identically. These experiments were repeated using A.T.P. instead of adenylic acid, and exactly the same sequence of events was seen (second panel of Fig. 1).

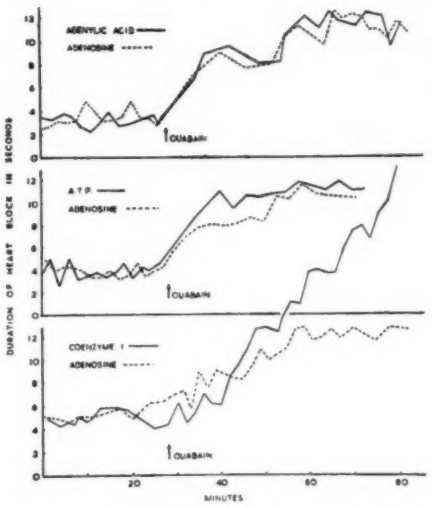

Fig. 1. Ouabain potentiation of heart block produced in the guinea-pig by adenyl compounds. Each adenyl compound was compared separately with adenosine by alternate injections through two cannulae tied into the left atrium. The ordinate shows the duration of the heart block (in seconds) produced by regular injections of each compound. A marked increase follows the commencement of a constant injection of ouabain (1·5 μg./kg./min.). Each point on the graphs represents the mean values from 3 separate experiments.

To compare coenzyme I with adenosine in this way, a dose level which produced initially equal responses was chosen. When the injection of ouabain was commenced, the response to coenzyme I increased in duration far more

rapidly than the adenosine response; the final degree of potentiation of the coenzyme I heart block was considerably greater. This is clearly shown in the bottom panel of Fig. 1, which again represents results obtained from 3 animals.

Potentiation of the action of adenosine by other cardiac glycosides.

In order to determine the relative activity of other cardiac glycosides in potentiating the adenosine heart block, it was first necessary to ascertain the effect of different rates of injection of the glycoside on the degree of this potentiation. Accordingly, four different rates of injection of ouabain were used: 0·35, 0·70, 1·40 and 2·80 μg./kg./min. At the two lower rates of infusion, the potentiation of the response took longer to develop, while at the highest rate of infusion, adenosine precipitated irregularities in the E.C.G. before the maximum degree of potentiation was reached. The rate of potentiation of the adenosine response by different concentrations of ouabain is shown in Fig. 2, from which

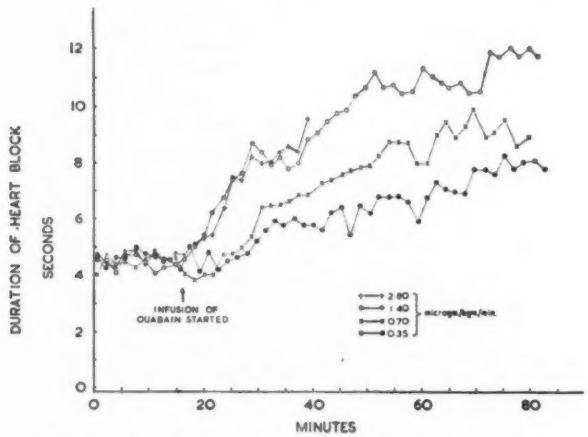

Fig. 2. The effect of the rate of injection of ouabain on the potentiation of the adenosine response. The duration of heart block produced by regular injections of adenosine is plotted against the time of infusion of ouabain solutions of four different concentrations. Each point is the mean of observations on two animals.

it can be seen that there is an optimum rate of infusion (for ouabain, 1·40 μg./kg./min.) above which the response to injections of adenosine is not further potentiated, but changes to electrocardiographic irregularities before the maximum degree of potentiation is seen. In the comparison of digoxin, digitoxin and lanatoside C, the optimum rate of infusion was determined for each glycoside (see legend to Fig. 3). As was the case with ouabain, the response to

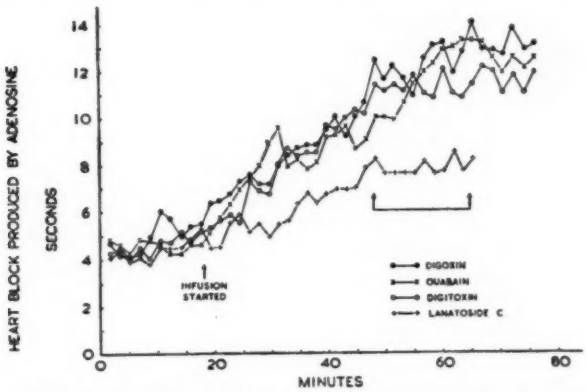

Fig. 3. The potentiation of the response to adenosine by different cardiac glycosides. Digoxin (5·6 μg./kg./min.), ouabain (1·4 μg./kg./min.), digitoxin (11·2 μg./kg./min.) and lanatoside C (5·6 μg./kg./min.) were each infused into three guinea-pigs which received regular injections of adenosine. Each point is the mean of the response in these three animals.

adenosine was always potentiated by these three cardiac glycosides. The results are shown graphically in Fig. 3. When all the glycosides were injected at their optimum rate, digoxin and digitoxin produced the same degree of potentiation as ouabain, but lanatoside C appeared to be less active. Statistical examination of the results in Fig. 3 showed that there was no significant difference between any of the responses to adenosine before administration of the cardiac glycosides. When the potentiation of the response was fully developed (indicated in Fig. 3 between arrows), the curves for digoxin, digitoxin and ouabain were not significantly different, whereas the curve for lanatoside C was significantly lower ($P=0·01$). With higher doses of each cardiac glycoside, which alone did not alter the configuration of the E.C.G., the response to injected adenosine changed to a period of ventricular extrasystoles.

DISCUSSION.

Though the general pharmacology of adenyl compounds in the guinea-pig has been described in detail, few comparisons have been made on a quantitative basis. This work has provided the opportunity of comparing the relative potencies of the adenyl compounds in producing heart block in the guinea-pig. Reid (1951) found that equimolar doses of A.T.P. and adenosine were equi-active in their effects on contraction of the guinea-pig auricle *in situ*. Wayne, Goodwin and Stoner (1949) found that equimolecular doses of Mg., A.T.P. and adenosine have the same actions on the E.C.G. of the guinea-pig. It is now shown that equimolar solutions of adenosine, adenylic acid and A.T.P. have exactly the same qualitative and quantitative actions on the guinea-pig heart.

Ouabain potentiated the heart block produced by adenosine, adenylic acid and A.T.P. in the guinea-pig to the same extent. It would be reasonable to suggest that any explanation of the mechanism of this potentiation must apply equally to all these compounds. Summation of the action of the adenyl compounds on the P–R interval with that of ouabain cannot explain this phenomenon, as the maximum degree of potentiation is seen at a stage when ouabain itself has not changed the E.C.G. Rand *et al.* (1955) have suggested that the potentiation of the action of adenosine by ouabain in the guinea-pig heart is due to the inhibition of an enzyme deaminating adenosine. Heart muscle from all species has been shown to contain an active adenosine deaminase (Ostern and Mann, 1933), while adenylic acid is only slowly deaminated (Kutscher and Sarreither, 1948; Conway and Cooke, 1939; Ostern and Mann, 1933). Similarly, A.T.P. is only slowly deaminated by heart muscle extracts which rapidly deaminate adenosine (Stafford and Rand, unpublished experiments). There does not appear to be any single enzyme in heart muscle which is responsible for the detoxification of all these substances. If the potentiating action of ouabain is really due to the inhibition of adenosine deaminase, it would be necessary to postulate a very rapid dephosphorylation of A.T.P. and adenylic acid to adenosine.

Coenzyme I has a qualitatively similar action to adenosine, but there is a marked difference in the slope of the dose response curves (Stafford and Rand, 1955). It is still not definitely known whether the pharmacological actions of coenzyme I depend upon the entire molecule, or whether it is broken down by a DPN-ase into a simpler adenyl compound and acts in this form. However, its action on the guinea-pig heart is potentiated by ouabain to a far greater extent than is the case with the other adenyl compounds, suggesting that some additional mechanism is involved in this potentiation, such as, perhaps, the inhibition of a DPN-ase. Govier *et al.* (1946) have postulated that digitoxin protects coenzyme I against degradation, but *in vitro* experiments by Spaulding and Graham (1947) have failed to confirm this.

Toxic irregularities in the E.C.G. are precipitated by four adenyl compounds when the dose of ouabain is still less than that which, if given alone, would be required to produce similar changes in the E.C.G. The marked resemblance between the toxic irregularity in the E.C.G. initiated by the adenyl compounds after small doses of cardiac glycosides and that produced at a higher dose level by the cardiac glycoside alone supports the suggestion made by Rand *et al.* (1955) that some of the pharmacological actions of the cardiac glycosides could be mediated by way of the naturally occurring adenyl compounds. The quantitatively identical action of ouabain, digoxin and digitoxin suggests that they act on the same system. We can offer no explanation here for the lower activity of lanatoside C.

SUMMARY.

Adenosine, adenylic acid and A.T.P. injected into the left atrium in equimolecular doses produce a quantitatively and qualitatively identical period of heart block in the guinea-pig. Small doses of ouabain, insufficient to have any other effect on the E.C.G., potentiate the effect of these three adenyl compounds to the same extent. Coenzyme I has a qualitatively similar action to adenosine on the guinea-pig E.C.G., but this effect is potentiated to a greater extent by ouabain than is the response to the other adenyl compounds.

Digoxin, digitoxin and lanatoside C in small doses also potentiated the effect of adenosine on the guinea-pig heart; lanatoside C is less active in this respect than the other cardiac glycosides. It has been shown that the rate of development of potentiation of the response to adenosine is proportional to the rate of infusion of the cardiac glycoside.

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REFERENCES.

Conway, E. J. and Cooke, R. (1939): Biochem. J., 33, p. 479.
Fischer, H. and Langemann, H. (1951): Helv. physiol. pharmacol. Acta, 9, p. 406.
Govier, W. M., Yanz, N. and Grelis, M. E. (1946): J. Pharm. exp. Ther., 88, p. 373.
Kutscher, W. and Sarreither, W. (1948): Klin. Wschr., 26, p. 698.
Loubatieres, A. (1951): J. Physiol., Paris, 43, p. 517.
Ostern, P. and Mann, T. (1933): Biochem. Z., 260, p. 326.
Rand, M., Stafford, A. and Thorp, R. H. (1955): J. Pharm. exp. Ther., 114, p. 119.
Reid, G. (1951): Austral. J. exp. Biol., 29, p. 243.
Spaulding, M. E. and Graham, W. D. (1947): J. biol. Chem., 170, p. 711.
Stafford, A. and Rand, M. (1955): Austral. J. exp. Biol., 33, p. 651.
Wayne, E. J., Goodwin, J. F. and Stone, H. B. (1949): Brit. Heart J., 11, p. 55.
Wollenberger, A. (1949): Pharmacol. Rev., 1, p. 311.

THE EFFECT OF YOHIMBINE AND OTHER DRUGS ON THE ISOLATED FROG SKIN POTENTIAL

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In an earlier paper (Shaw, Holman and Mackenzie, 1955), it had been shown that yohimbine was without effect on the resting potential of a single muscle fibre but did affect the action potential of this preparation. In the same paper evidence was presented to suggest that yohimbine affected transport of sodium ions in nerve during activity.

Ussing (1951) has said that the salt uptake by frog skin could be explained by the active transport of Na ions only. It would, therefore, be of interest to investigate the action of yohimbine on the skin potential and to compare it with the effect on muscle membrane potential. At the same time the influence of yohimbine on the effect of adrenaline and noradrenaline on the potential was investigated, in view of the well-known antagonism between these drugs.

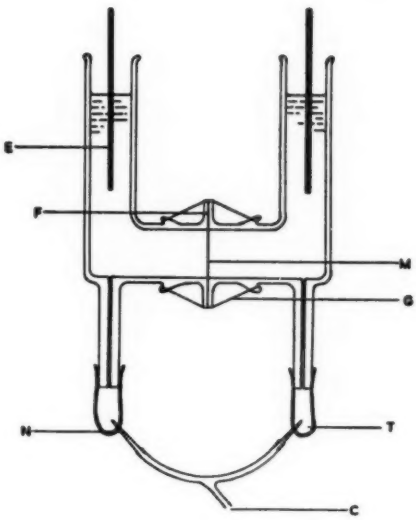

Fig. 1. Apparatus for the measurement of membrane potentials.

E—Ag/AgCl electrodes.

F—Ground glass faces.

M—Membrane—frog skin or cellophane.

G—Rubber bands keeping faces firmly and evenly pressed together.

T—Rubber teats which can be removed to allow solutions to drain from apparatus.

N—Syringe needles.

C—Plastic tubing whose diameter is adjusted to give equal bubbling of gas in either arm of apparatus. Carbogen, oxygen or nitrogen passed through C from cylinder.

METHODS.

The apparatus was arranged as in Fig. 1. The potentials were measured by a pen recording galvanometer which was driven by a D.C. amplifier, as in Fig. 2. If the stirring were not efficient the potential would decrease. Good stirring was obtained by allowing carbogen (5 p.c. CO_2 in oxygen) to bubble through the solution.

Ringer (modified): In one litre of water: NaCl, 6·7 gm.; KCl, 0·14 gm.; $NaHCO_3$, 0·20 gm.; NaH_2PO_4, 0·01 gm.; $CaCl_2$, 0·12 gm.; Glucose, 2·00 gm.

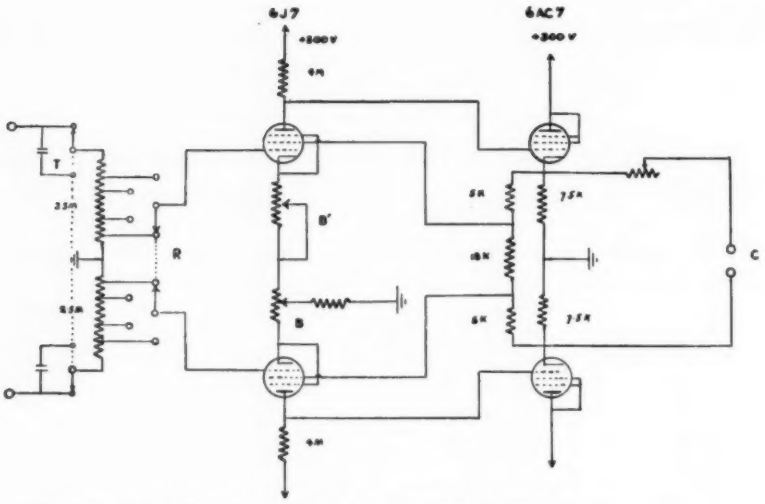

Fig. 2. Amplifier used with C.M.E.'s and in frog skin work.
R—5-position switch for altering range of operation from 0–100 mV to 0–2V.
T—Test switch for grounding input.
B—Coarse balance (inside case).
B^1—Balance.
C—Output to chart recorder.
Amplifier operated by conventional power supply (V.R. regulated, and running from 50 volt-amp. constant voltage transformer).
Note that the 1st stage is constructed according to the "starvation" principle.

RESULTS.

Effect of calcium ions. The modified Ringer was made up with different Ca concentrations, i.e., 0, 1, 2, 4, 8 meq./litre. These solutions were applied in turn, each for ¼ hour to the skin after it had attained a steady potential. Four skins were studied—3 males and 1 female, and in each case the results shown in Table 1 were obtained.

Hence it appears that Ca is not essential for the maintenance of potential over short periods but an excess leads to a rapid increase in potential. It is

TABLE 1.

Concentration	Effect
0 meq/litre	No significant difference
1 meq/litre	No significant difference
2 meq/litre	No significant difference
4 meq/litre	Rise
8 meq/litre	Very large rise which took place very rapidly

interesting to note that in one case (that of a female frog) a potential of 175 mv. was reached with excess Ca.

Adrenaline and noradrenaline. Effects were obtained only when the drugs were added to the inside of the skin. Concentrations of $1:10^5$ were used. The results obtained are shown in Tables 2 and 3.

TABLE 2.

Adrenaline

	Potential at time of dose (m.V.)	Response			Recovery	
		Time (to reach max.) min.	Rise to max. value (m.V.)	Fall (m.V.)	Time min.	Value (m.V.)
Males	28 falling	9	10	8	31	38
	51 steady	—	—	16	30	29
	31 falling	3	14	35	21	26
	27 steady	2	7	32	no recovery recorded	
	80 falling	—	—	50	29	51
	10 falling	—	—	5	30	8
	12·5 steady	4	6	8	no recovery recorded	
	13 steady	5	1·5	4·5	no recovery recorded	
	42 steady	4	3	36	30	36
	12 falling	3	3·5	3·5	12	9
Females	70 steady	—	—	46	40	45
	49 steady	—	—	1·5	20	45·5
	46·55 steady	—	—	5	20	45
	49 steady	3	2	10	30	38·5
	30 steady	7	11	26	30	20
	94 steady	—	—	48	25	63
	51 falling	15	40	no fall recorded	20	81
	80 falling	12	11	no fall recorded	20	96
	48 steady	—	—	36	no recovery recorded	
	10 falling	3	19	23	no recovery recorded	

TABLE 3.

Nor-Adrenaline (Levophed).

	Potential at time of dose (m.V.)	Response			Recovery	
		Time (to reach max.) min.	Rise to max. value (m.V.)	Fall (m.V.)	Time min.	Value (m.V.)
Males	39 falling	4	27	38	32	31
	38 steady	—	—	12	21	40
	38 steady	5	2	16	30	29
	22 steady	3	8	15	30	10
	10 steady	2	14	15	30	17
Females	35 steady	8	14	9	20	21
	49 steady	—	—	14	30	52
	52 steady	—	—	13	20	45

In general the effect of the drugs is to cause a rise in potential followed by a fall.

Yohimbine. The results are shown in Table 4; they indicate that yohimbine causes an elevation of potential.

Adrenaline and yohimbine. To test whether yohimbine had any action on the adrenaline effect observed above, the following method was used.

As soon as a steady potential was reached, a dose of adrenaline ($1:10^5$) was given. About 20 minutes were allowed for recovery after the adrenaline was washed out and the yohimbine Ringer was added. When the potential reached a steady value (about 30 minutes later) adrenaline was given again so as to enable comparison between the responses of the same skin. Unfortunately, owing to difficulties in maintaining a steady potential for a period of over 2 hours, it was not always possible to do an adrenaline test before adding the yohimbine and allowing the skin to become fully yohimbinized. Some representative samples of these experiments are set out in Table 5.

In all cases yohimbine appeared to lessen the effects of adrenaline on the isolated skin potential.

TABLE 4.

	Dose of Yohimbine	Potential at time of dose (m.V.)	Response		Further Comments
			Time to max. min.	Rise (m.V.)	
Females	2×10^{-3}	43 falling	4	20	Remained at 40 for 10 mins.
	10^{-3}	44 falling quickly	10	1	*Yoh. steadied fast falling off*—20mins. later potential 29 m.V.
	10^{-3}	63 steady	5	8	34 mins. later—49 m.V.
	10^{-3}	64 steady	5	15	35 mins. later—63 m.V.
	2×10^{-4}	46 steady	10	5	
Males	2×10^{-3}	43 falling	4	20	Steady at 63 for 10 mins.
	2×10^{-3}	31 falling	2	4	Steady at 35 for 8 mins.
	10^{-3}	10 steady	10	11	Steady at 20 for 10 mins.
	10^{-3}	11 falling	Slower fall only observed		
	10^{-3}	13 steady	7	7	After 20 mins.—14 m.V.
	10^{-3}	30 steady	6	19	After 40 mins.—20 m.V.
	2×10^{-3}	65 steady	5	39	After 60 mins.—70 m.V.

TABLE 5.

Treatment		Response					Further Remarks
		Pot. at dose pt. m.V.	Rise		Fall		
			Time (mins)	Amount (m.V.)	Time (mins)	Amount (m.V.)	
(i)	Adr.	70 st.	—	—	5	46	40 mins. later pot. 47 m.V. Only Yohimbinized for 13 mins.
	Yoh.-Adr.	61 rising	—	—	5	30	Not fully Yohimbinized.
Females	Adr.	94 st.	—	—	4	48	Recovered to 63 m.V. before Yoh. added—after 35 mins. in continued to fall after w.o.
	Yoh.-Adr.	49 st.	—	—	4	13	Yoh. steady at 49 m.V.
	Adr.	70 st.	4	2	8	45	Recovered to 70 with Yoh. in 45 mins.
	Yoh.-Adr.	72 st.	—	—	16	16	Recovered to 65 m.V.
	Adr.	34 fall.	—	—	6	24	Let recover in Yoh.—50 mins.—20 m.V.
	Yoh.-Adr.	20 st.	—	—	6	6	Recovery not recorded above 10 m.V.
Males	Adr.	51 st.	—	—	4	27	18 mins. later—42 m.V. after Yoh. added 56 mins. later 20 m.V. steady.
	Yoh.-Adr.	20 st.	—	—	6	7	
(ii)	Yoh.-Adr.	70 st.	—	—	6	7	Recovered in 20 mins. 70 m.V.
Females	Yoh.-Adr.	20 fall.	2	—	falling off rate decreased slightly.		
	Yoh.-Adr.	57 fall.	—	—	7	8	Steady at 57 m.V. for 4 mins. before fall—56 m.V. after 20 mins.
Males	Yoh.-Adr.	14 fall.	—	—	4	6	10 mins. later, 10 m.V.
	Yoh.-Adr.	70 st.	—	—	7	10	10 mins. later 65 m.V.

st. = stationary fall. = falling

Sodium azide and hydroxylamine. These drugs were used in concentrations of $1:10^3$ and $1:10^4$. In both cases, they produced a rapid fall in the skin potential to zero or a few m.v. The rate of fall varied with different skins. The average of 5 experiments with $1:10^3$ sodium azide showed that the potential was abolished or reached a minimum in about ½ hour. In several, but not all cases the hydroxylamine effect was reversible. In two cases out of eight, the potential recovered, even in the presence of the drug. These results tend to substantiate the claim that the skin potential has a metabolic background.

DISCUSSION.

It is evident from these results that yohimbine lessens the effect of adrenaline on the frog skin potential and so another example of this well-known antagonism is furnished.

Ussing (1951) was able to show, in his "short circuit" experiments, that adrenaline increased the sodium uptake by an increase in C_{Na+}[1] in spite of a decrease in active transport potential. Both these factors would tend to lower the skin potential, a fact which we observed in almost every experiment. The initial increase in potential must have been due to another factor. This can only be guessed at from present evidence, but adrenaline may have had an initial stimulating effect on the active transport mechanism, or decreased the permeability to chloride ion. Yohimbine could have counteracted the effect of adrenaline by decreasing C_{Na+}. These suggestions can only be verified by further experimentation, with the combined use of electrical and tracer methods.

Perhaps of more importance is the fact that yohimbine can influence the potential, either alone or in combination with adrenaline. The exact nature of the skin potential is not known, but Ussing (1951) has shown that the accumulation of salt by the skin is due to the active transport of Na ions (the chloride presumably following passively). It would not therefore be incorrect to assume that portion of the potential at least is due to this Na movement.

In an earlier paper (Shaw *et al.*, 1955), the suggestion was made that yohimbine influenced only the passage of Na ions across a cell boundary when that cell was in an active state (i.e., contraction or conduction). It would appear that the results given in this paper tend to substantiate this claim if one allows that the skin potential is connected with the active transport of Na ions.

SUMMARY.

Calcium ions produce a rise in frog skin potential.

Adrenaline and noradrenaline in general produce a rise in potential followed by a fall.

Yohimbine brings about an elevation of potential.

The fall in potential produced by adrenaline is lessened by yohimbine.

Sodium azide abolishes the potential.

The postulate that yohimbine affects only active movements of sodium ions is discussed.

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REFERENCES.

Shaw, F. H., Holman, Mollie E. and Mackenzie, J. G. (1955): Austral. J. exp. Biol. In press.
Ussing, H. H. and Zerahn, K. (1951): Acta physiol. Scand., 23, p. 110.

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[1] C_{Na+} represents the conductivity of the skin to sodium ions.